

**COMPOSITIONS AND METHODS RELATING TO NOVEL
COMPOUNDS AND TARGETS THEREOF**

This application is a Continuation in Part of U.S. Patent Application Serial
5 No.: 10/427,211, filed May 1, 2003, which is a continuation in part of U.S. Patent
Application Serial No.: 10/217,878, filed August 13, 2002, which is a continuation of
U.S. Patent Application Serial No.: 09/767,283, filed January 22, 2001, which is a
continuation of U.S. Patent Application Serial No.: 09/700,101, filed November 8,
2000, which is the National entry of PCTUS00/11599 filed April 27, 2000, which
10 claims priority to U.S. Provisional Application Serial No.: 60/131,761, filed April 30,
1999, to U.S. Provisional Application Serial No.: 60/165,511, filed November 15,
1999, and to U.S. Provisional Application Serial No.: 60/191,855, filed March 24,
2000. U.S. Application Serial No.: 10/217,878 also claims priority to U.S.
Provisional Application Serial No.: 60/312,560, filed August 15, 2001, and to U.S.
15 Provisional Application Serial No.: 60/313,689, filed August 20, 2001, and to U.S.
Provisional Application Serial No.: 60/396,670, filed July 18, 2002. Each
aforementioned application is specifically incorporated herein by reference in its
entirety.

This invention was supported in part with NIH grants GM46831 and AI47450.
20 The United States government may have rights in this invention.

FIELD OF THE INVENTION

The present invention relates to novel chemical compounds, methods for their
discovery, and their therapeutic use. In particular, the present invention provides
25 benzodiazepine derivatives and related compounds and methods of using
benzodiazepine derivatives and related compounds as therapeutic agents to treat a
number of conditions associated with the faulty regulation of the processes of
programmed cell death, autoimmunity, inflammation, hyperproliferation, and the like.

30 **BACKGROUND OF THE INVENTION**

Multicellular organisms exert precise control over cell number. A balance
between cell proliferation and cell death achieves this homeostasis. Cell death occurs

in nearly every type of vertebrate cell via necrosis or through a suicidal form of cell death, known as apoptosis. Apoptosis is triggered by a variety of extracellular and intracellular signals that engage a common, genetically programmed death mechanism.

5 Multicellular organisms use apoptosis to instruct damaged or unnecessary cells to destroy themselves for the good of the organism. Control of the apoptotic process therefore is very important to normal development, for example, fetal development of fingers and toes requires the controlled removal, by apoptosis, of excess interconnecting tissues, as does the formation of neural synapses within the
10 brain. Similarly, controlled apoptosis is responsible for the sloughing off of the inner lining of the uterus (the endometrium) at the start of menstruation. While apoptosis plays an important role in tissue sculpting and normal cellular maintenance, it is also the primary defense against cells and invaders (*e.g.*, viruses) which threaten the well being of the organism.

15 Not surprisingly many diseases are associated with dysregulation of the process of cell death. Experimental models have established a cause-effect relationship between aberrant apoptotic regulation and the pathenogenicity of various neoplastic, autoimmune and viral diseases. For instance, in the cell mediated immune response, effector cells (*e.g.*, cytotoxic T lymphocytes “CTLs”) destroy virus-infected
20 cells by inducing the infected cells to undergo apoptosis. The organism subsequently relies on the apoptotic process to destroy the effector cells when they are no longer needed. Autoimmunity is normally prevented by the CTLs inducing apoptosis in each other and even in themselves. Defects in this process are associated with a variety of autoimmune diseases such as lupus erythematosus and rheumatoid arthritis.

25 Multicellular organisms also use apoptosis to instruct cells with damaged nucleic acids (*e.g.*, DNA) to destroy themselves prior to becoming cancerous. Some cancer-causing viruses overcome this safeguard by reprogramming infected (transformed) cells to abort the normal apoptotic process. For example, several human papilloma viruses (HPVs) have been implicated in causing cervical cancer by
30 suppressing the apoptotic removal of transformed cells by producing a protein (E6) which inactivates the p53 apoptosis promoter. Similarly, the Epstein-Barr virus (EBV), the causative agent of mononucleosis and Burkitt's lymphoma, reprograms

infected cells to produce proteins that prevent normal apoptotic removal of the aberrant cells thus allowing the cancerous cells to proliferate and to spread throughout the organism.

Still other viruses destructively manipulate a cell's apoptotic machinery
5 without directly resulting in the development of a cancer. For example, the destruction of the immune system in individuals infected with the human immunodeficiency virus (HIV) is thought to progress through infected CD4⁺ T cells (about 1 in 100,000) instructing uninfected sister cells to undergo apoptosis.

Some cancers that arise by non-viral means have also developed mechanisms
10 to escape destruction by apoptosis. Melanoma cells, for instance, avoid apoptosis by inhibiting the expression of the gene encoding Apaf-1. Other cancer cells, especially lung and colon cancer cells, secrete high levels of soluble decoy molecules that inhibit the initiation of CTL mediated clearance of aberrant cells. Faulty regulation of the apoptotic machinery has also been implicated in various degenerative conditions and
15 vascular diseases.

It is apparent that the controlled regulation of the apoptotic process and its cellular machinery is vital to the survival of multicellular organisms. Typically, the biochemical changes that occur in a cell instructed to undergo apoptosis occur in an orderly procession. However, as shown above, flawed regulation of apoptosis can
20 cause serious deleterious effects in the organism.

There have been various attempts to control and restore regulation of the apoptotic machinery in aberrant cells (*e.g.*, cancer cells). For example, much work has been done to develop cytotoxic agents to destroy aberrant cells before they proliferate. As such, cytotoxic agents have widespread utility in both human and
25 animal health and represent the first line of treatment for nearly all forms of cancer and hyperproliferative autoimmune disorders like lupus erythematosus and rheumatoid arthritis.

Many cytotoxic agents in clinical use exert their effect by damaging DNA (*e.g.*, cis-diaminodichroplatanim(II) cross-links DNA, whereas bleomycin induces
30 strand cleavage). The result of this nuclear damage, if recognized by cellular factors like the p53 system, is to initiate an apoptotic cascade leading to the death of the damaged cell.

However, existing cytotoxic chemotherapeutic agents have serious drawbacks. For example, many known cytotoxic agents show little discrimination between healthy and diseased cells. This lack of specificity often results in severe side effects that can limit efficacy and/or result in early mortality. Moreover, prolonged
5 administration of many existing cytotoxic agents results in the expression of resistance genes (*e.g.*, *bcl-2* family or multi-drug resistance (MDR) proteins) that render further dosing either less effective or useless. Some cytotoxic agents induce mutations into p53 and related proteins. Based on these considerations, ideal
10 cytotoxic drugs should only kill diseased cells and not be susceptible to chemo-resistance.

One strategy to selectively kill diseased cells is to develop drugs that selectively recognize molecules expressed in diseased cells. Thus, effective cytotoxic chemotherapeutic agents, would recognize disease indicative molecules and induce
15 (*e.g.*, either directly or indirectly) the death of the diseased cell. Although markers on some types of cancer cells have been identified and targeted with therapeutic antibodies and small molecules, unique traits for diagnostic and therapeutic
exploitation are not known for most cancers. Moreover, for diseases like lupus, specific molecular targets for drug development have not been identified.

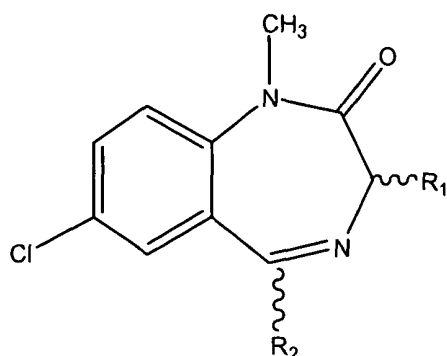
What are needed are improved compositions and methods for regulating the
20 apoptotic processes in subjects afflicted with diseases and conditions characterized by faulty regulation of these processes (*e.g.*, viral infections, hyperproliferative autoimmune disorders, chronic inflammatory conditions, and cancers).

SUMMARY

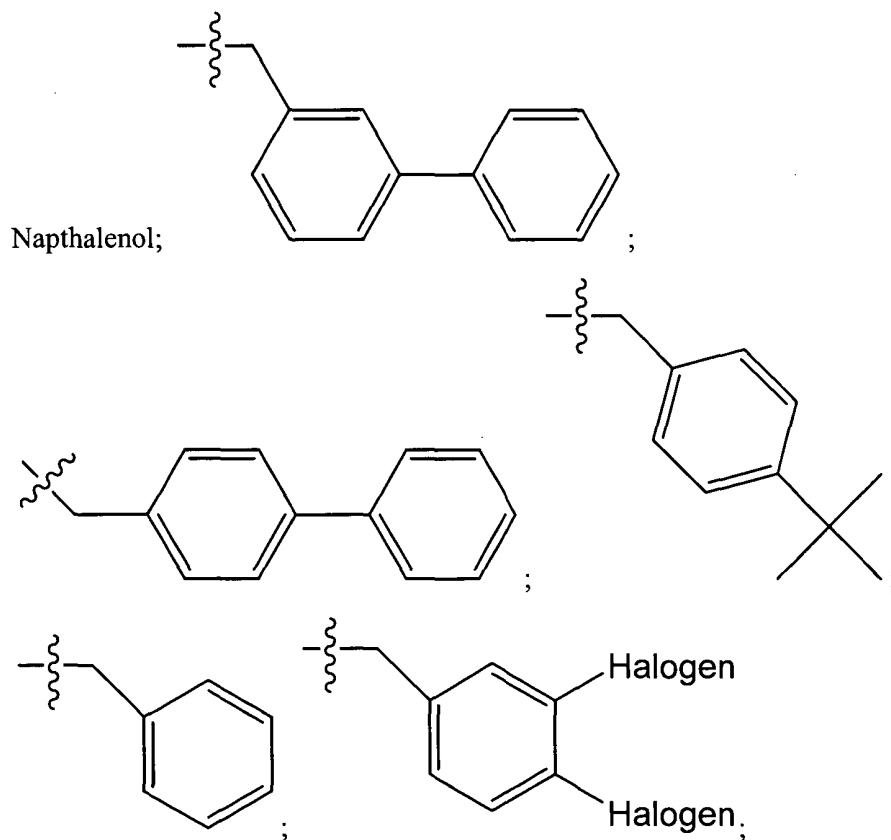
25 The present invention provides novel compounds that find use in treating a number of diseases and conditions and that find use in research, compound screening, and diagnostic applications. The present invention also provides uses of these novel compounds, as well as the use of known compounds, that elicit particular biological responses (*e.g.*, compounds that bind to particular target molecules and/or cause
30 particular cellular events). Such compounds and uses are described throughout the present application and represent a diverse collection of compositions and applications.

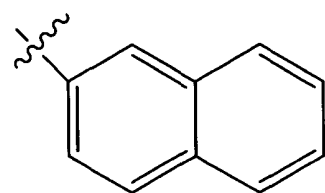
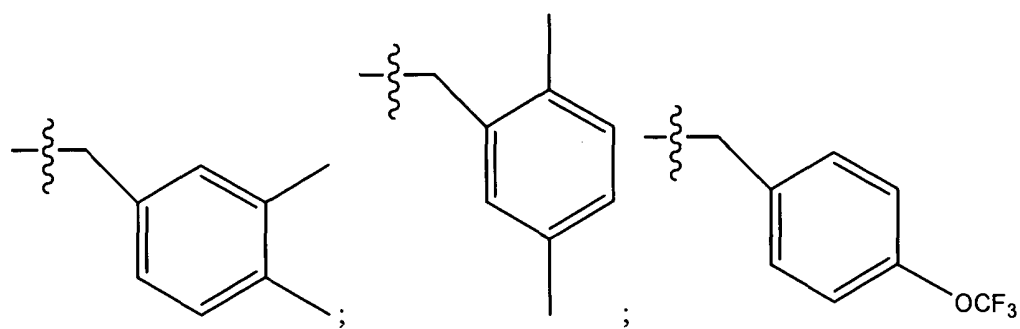
Certain preferred compositions and uses are described below. The present invention is not limited to these particular compositions and uses.

The present invention provides a number of useful compositions as described throughout the present application. Certain preferred embodiments of the present
 5 involve compositions include a composition comprising the following formula:

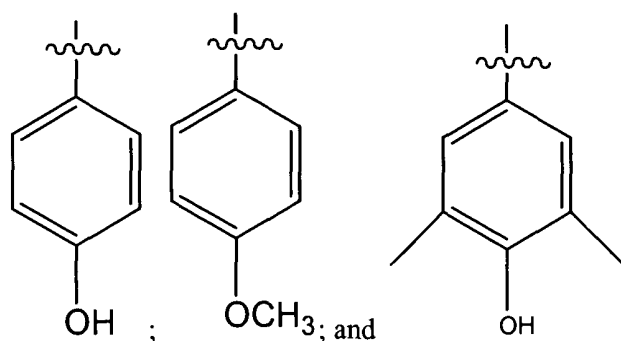
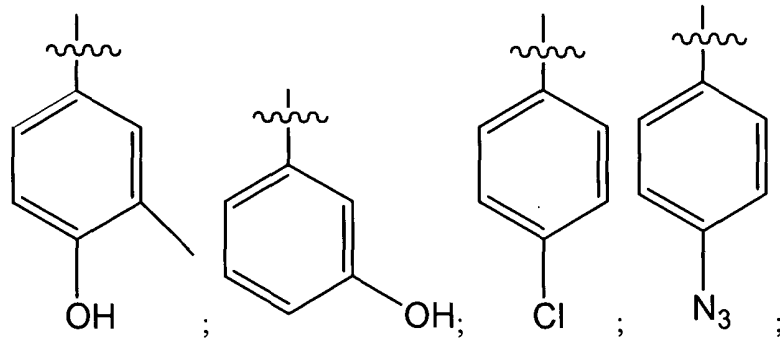


wherein R_1 is selected from naphthalanine; phenol; 1-Napthalenol; 2-



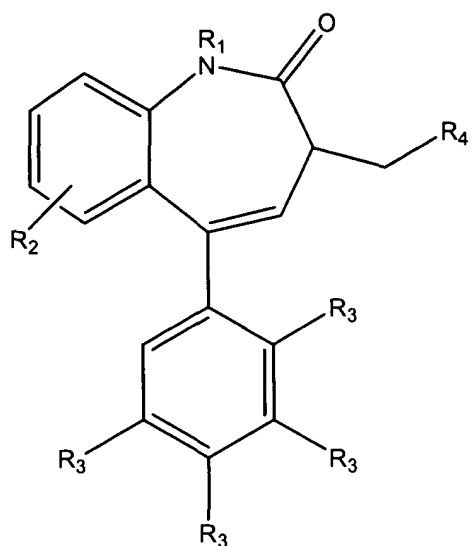


and quinolines; wherein R_2 is selected from the group consisting of:

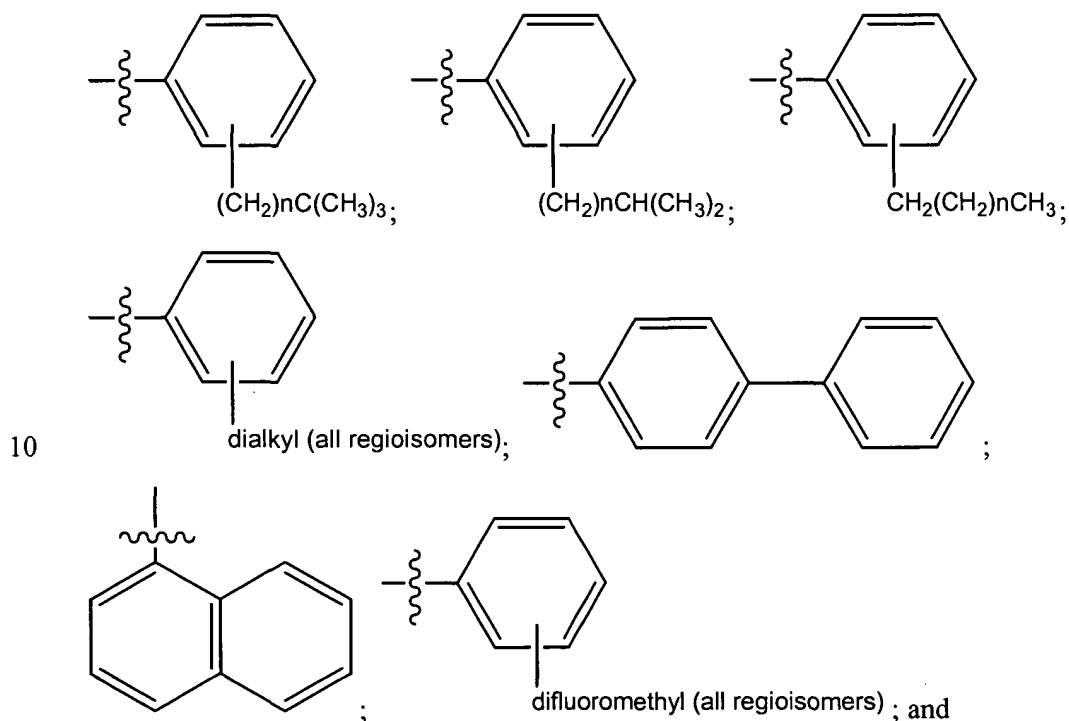


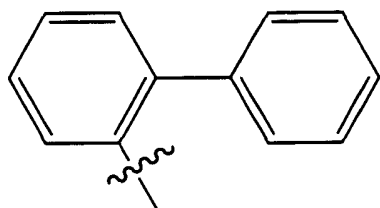
5 OH ; OCH_3 ; and OH ; and wherein R_1 and R_2 include both R or S enantiomeric forms and racemic mixtures. .

Other preferred embodiments of the present involve compositions include a composition comprising the following formula:



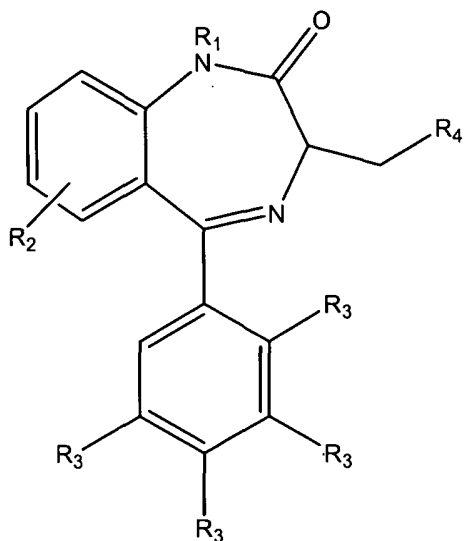
- wherein R1 is selected from H, alkyl, or substituted alkyl; wherein R2 is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl, a substituted amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, a heterocyclic; wherein R3 is selected from H, alkyl, or substituted alkyl, and wherein at most one substituent is a hydroxyl subgroup; wherein R4 is selected from



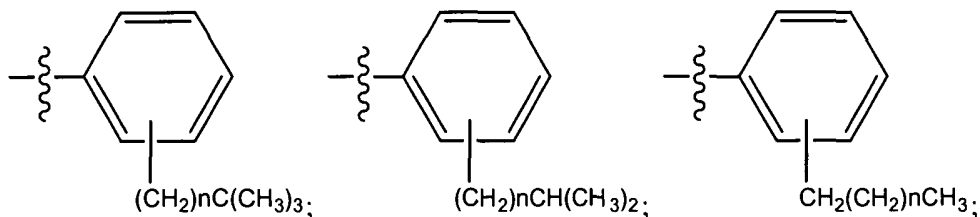


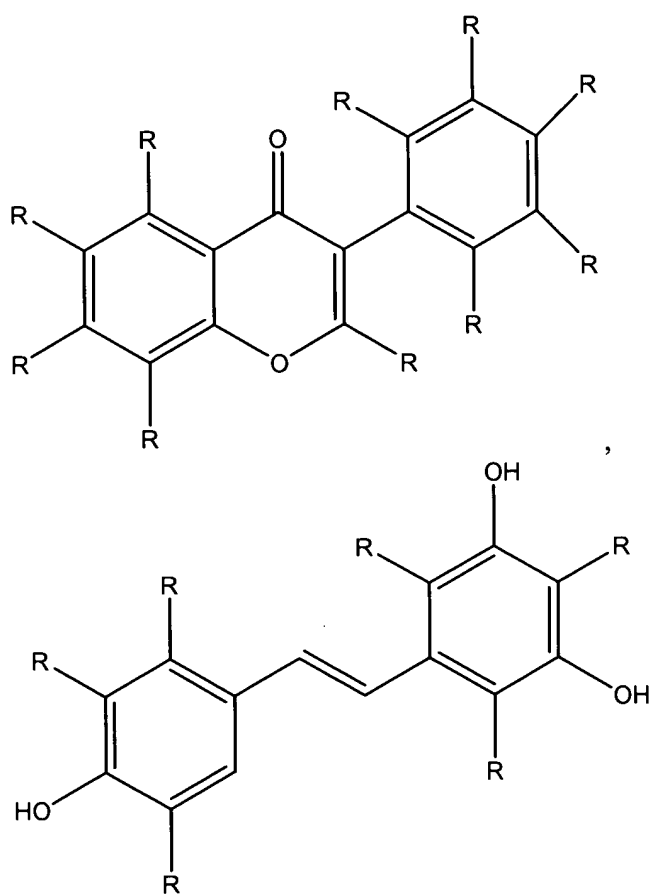
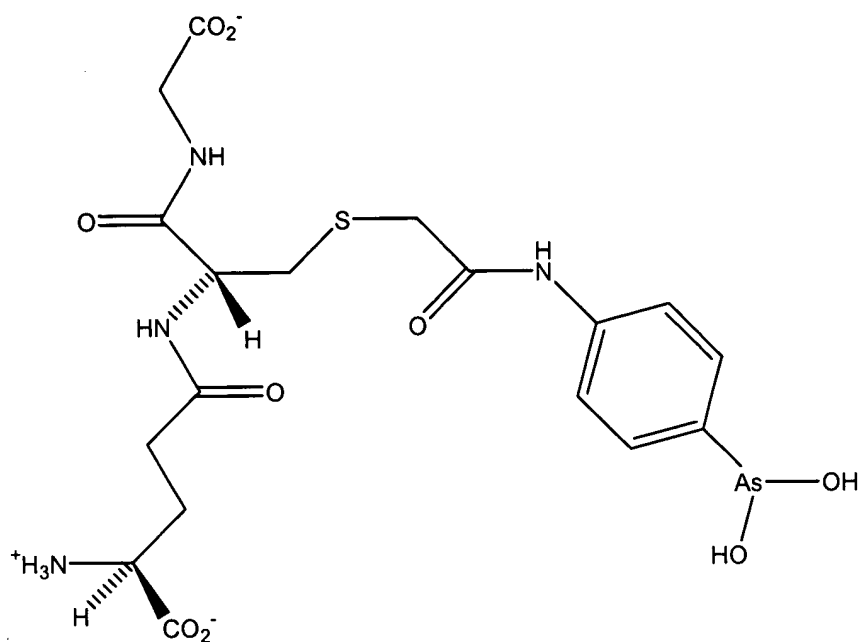
wherein $n = 0 - 5$; and wherein R_1 , R_2 , R_3 and R_4 include both R or S enantiomeric forms and racemic mixtures.

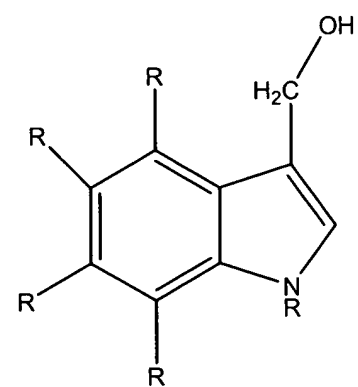
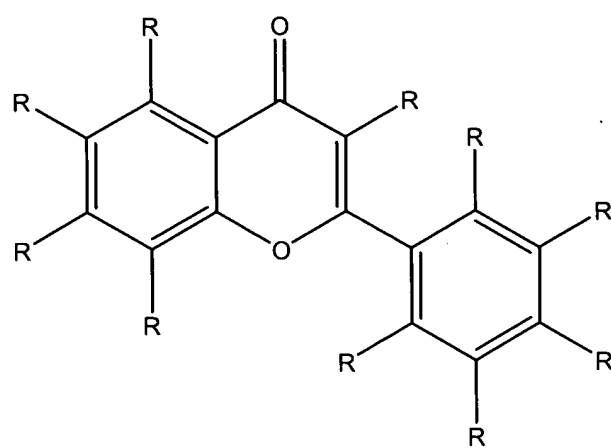
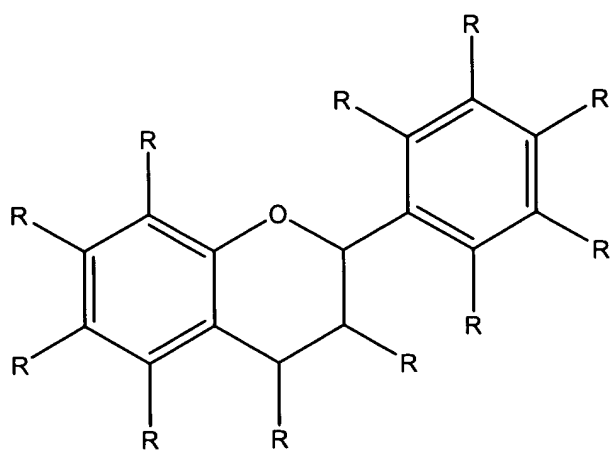
Still other preferred embodiments of the present involve compositions include a composition comprising the following formula:



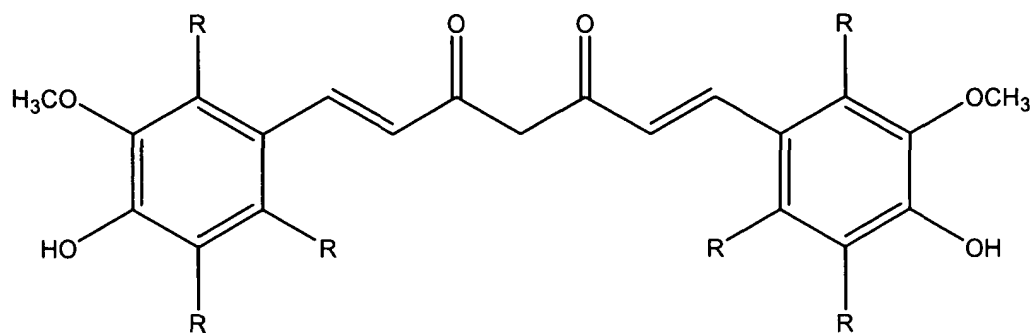
5 wherein R_1 is selected from H, alkyl, or substituted alkyl; wherein R_2 is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl, a substituted amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group
10 consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, a heterocyclic; wherein R_3 is selected from H, alkyl, or substituted alkyl, and wherein at most one substituent is a hydroxyl subgroup; wherein R_4 is selected from







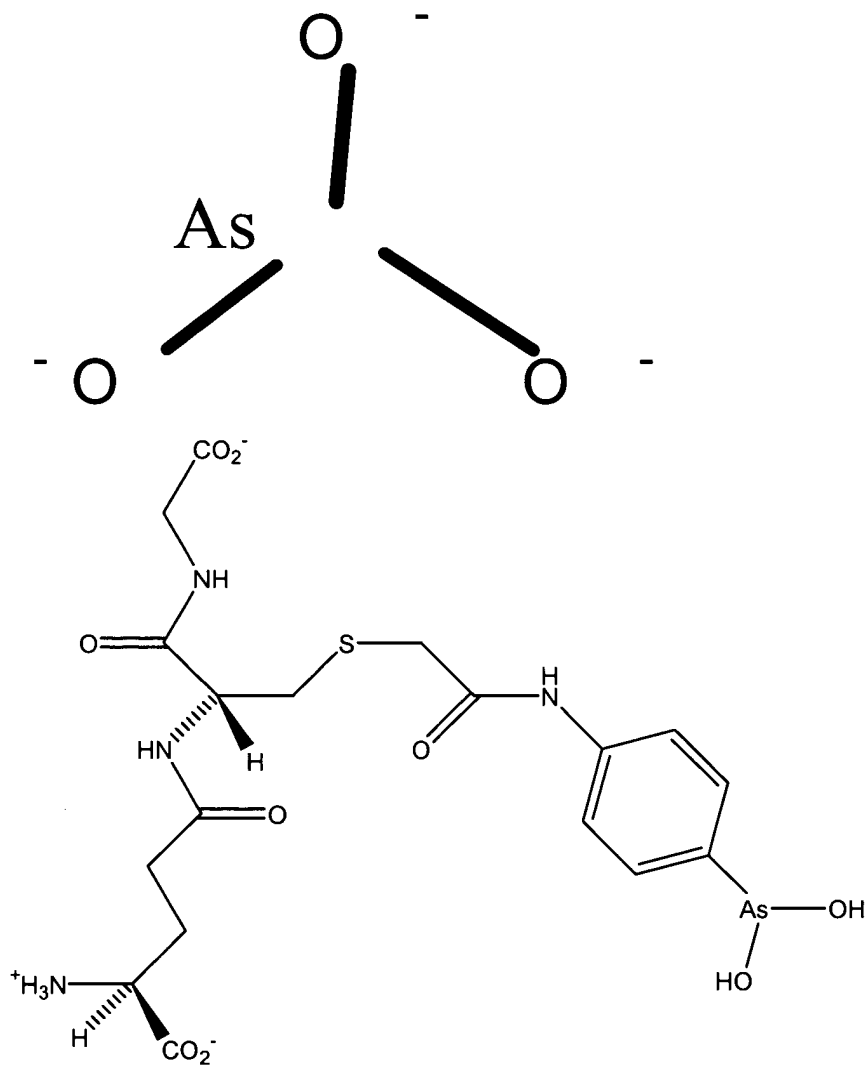
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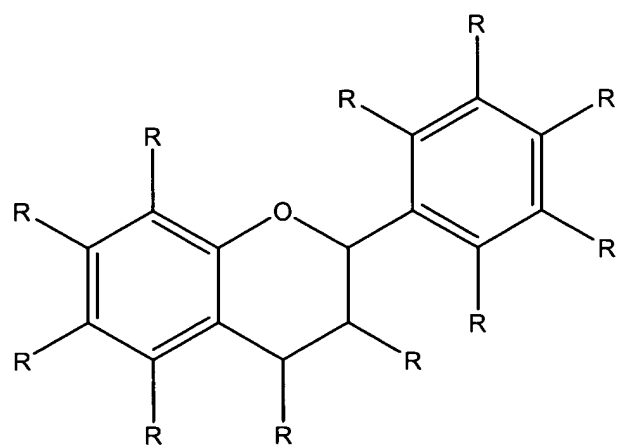
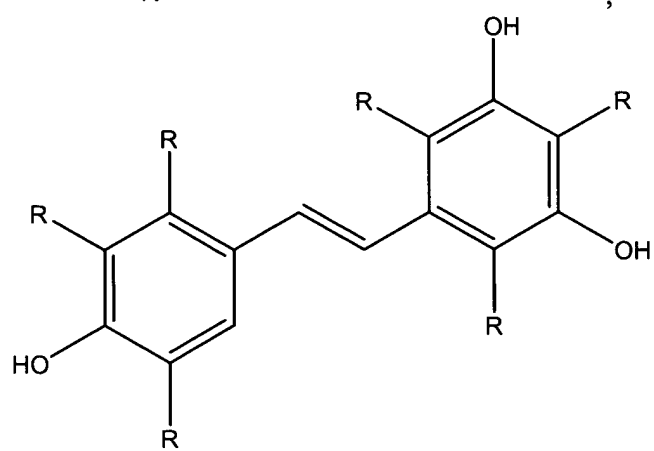
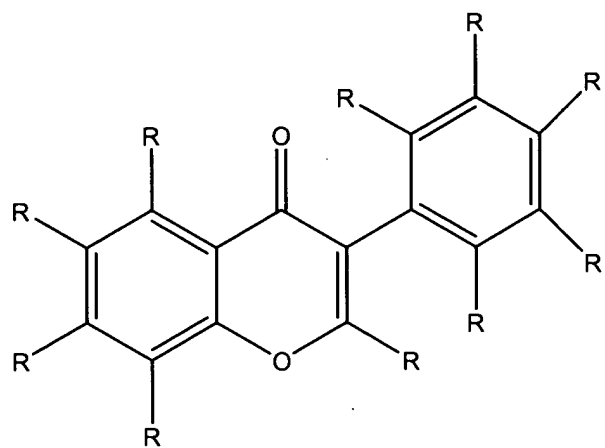


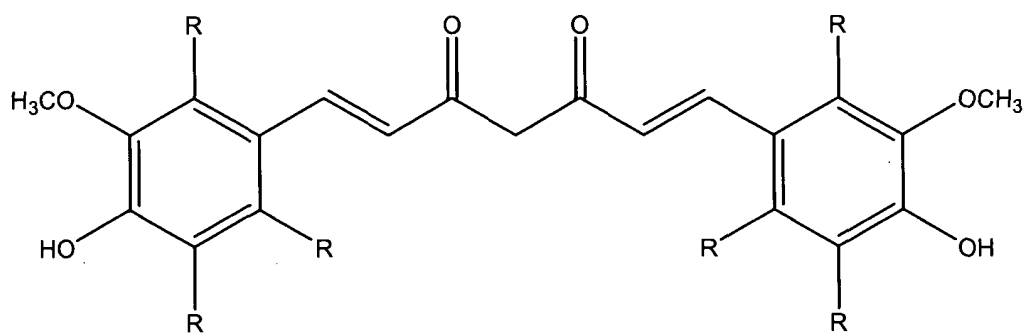
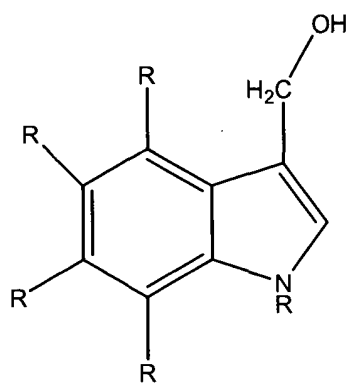
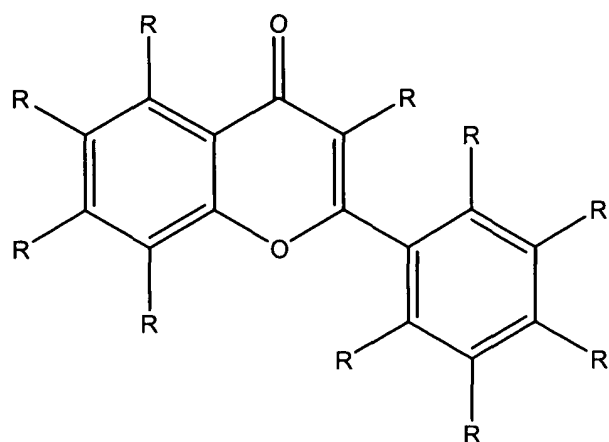
- wherein R is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl, a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having
- 5 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic; and such a composition is administered to the subject.

In still other preferred embodiments, the present invention provides compositions and methods for regulating cellular proliferation. In such embodiments, the present invention provides a subject and a composition comprising a formula

5 selected from:





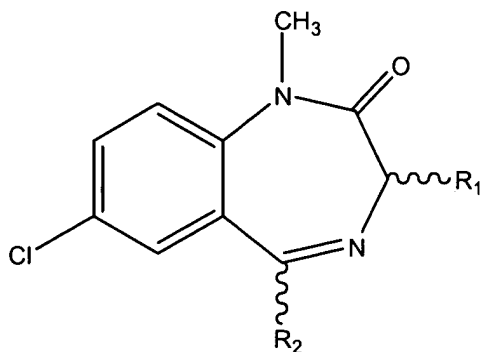


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wherein R is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl, a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a
 10 cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic; and the composition is administered to the subject.

The present invention provides a number of methods for influencing the fate of cells, tissues, and organisms. Certain preferred embodiments of the present involve

methods for regulating cell death. In such embodiments, the present invention provides target cells having mitochondria and a composition comprising the following formula:

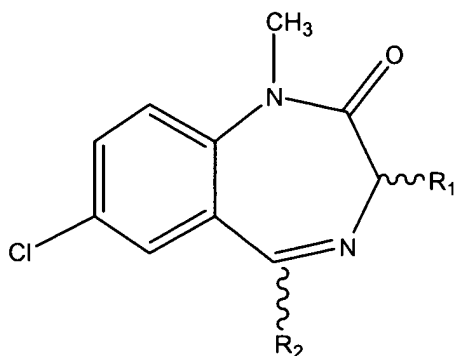


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wherein R1 comprises a hydrophobic aromatic group larger than benzene; wherein R2 comprises a phenolic hydroxyl group; and wherein R₁, and R₂ include both R or S enantiomeric forms and racemic mixtures. In additional embodiments, the cells are exposed to the composition under conditions such that said composition binds to the oligomycin sensitivity conferring protein so as to increase superoxide levels or alter cellular ATP levels in said cells.

In other embodiments, target cells are *in vitro* cells. In other embodiments, the target cells are *in vivo* cells. In still other embodiments, the target cells are *ex vivo* cells. In yet other embodiments, the target cells are cancer cells. In some embodiments, the target cells are selected from the group consisting of B cells, T cells, and granulocytes.

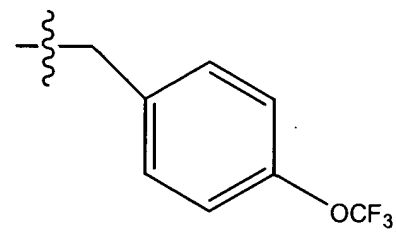
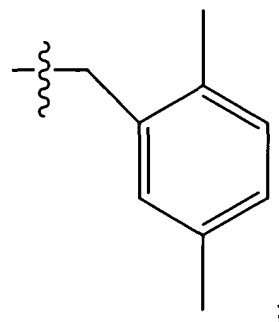
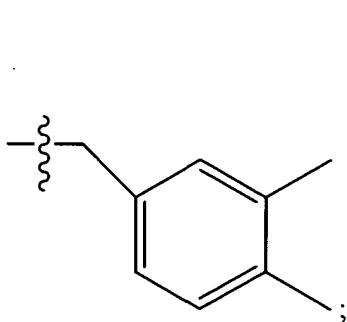
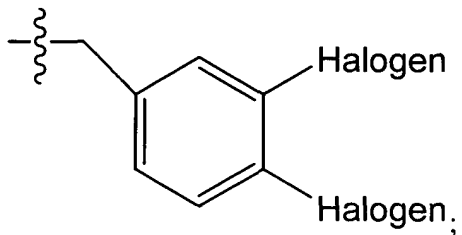
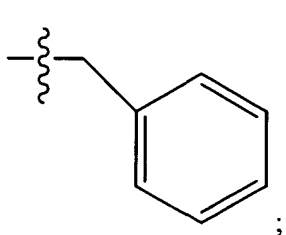
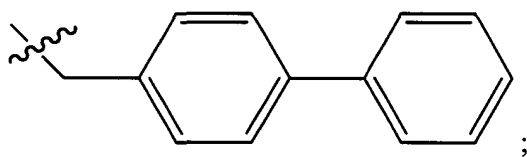
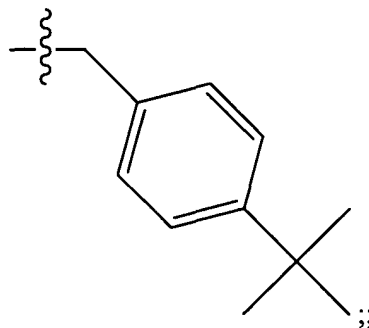
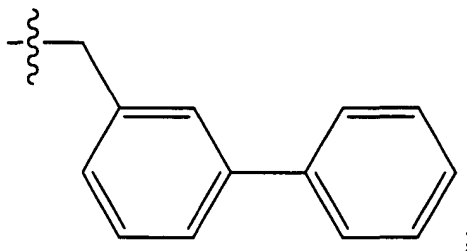
In other embodiments used in the regulation of cellular death, the present invention also provides the following compositions:



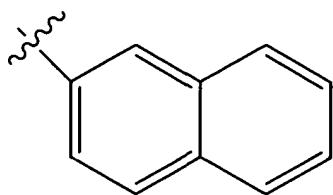
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wherein R₁ is selected from group consisting of: naphthalalanine; phenol; 1-

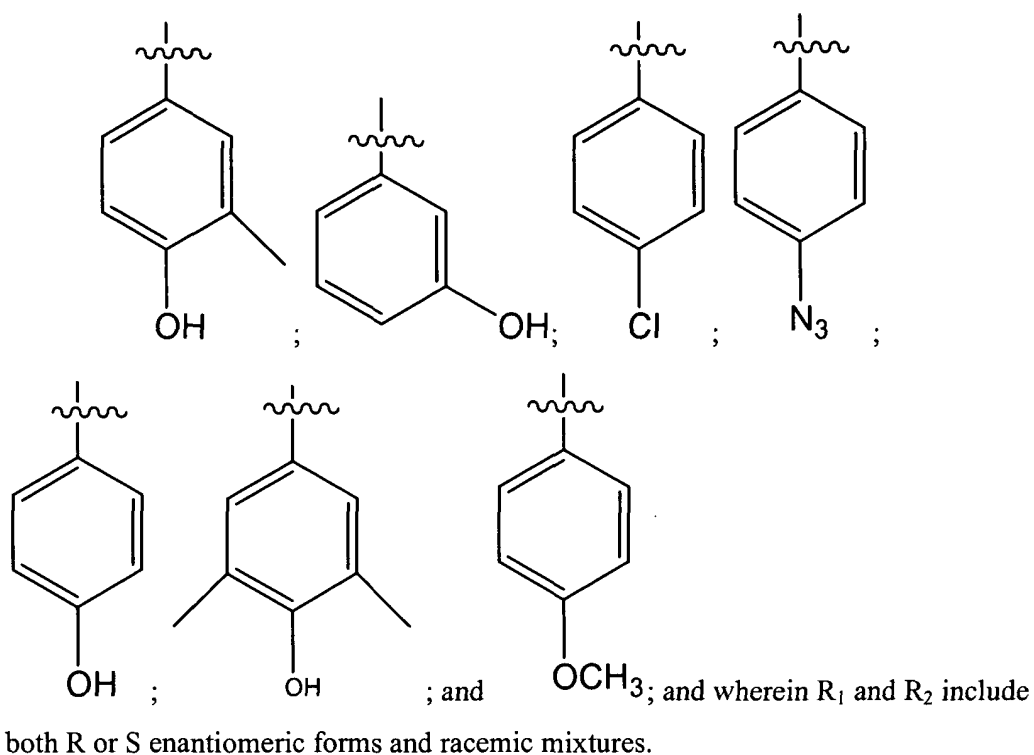
Napthalenol; 2-Napthalenol;



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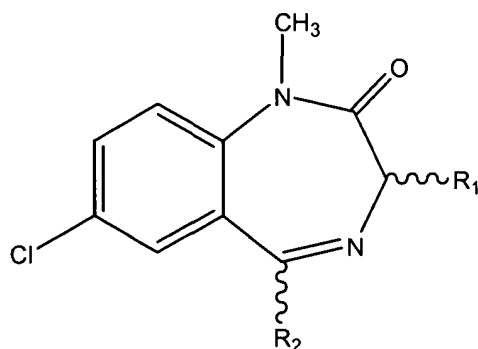
and quinolines; wherein R₂ is selected from the group consisting of:



5 In preferred embodiments wherein the present invention regulates cellular death, exposure of the composition to target cells results in an increase in cell death of the target cells.

The present invention also provides methods and compositions for regulating cellular proliferation. In such embodiments, the present invention provides

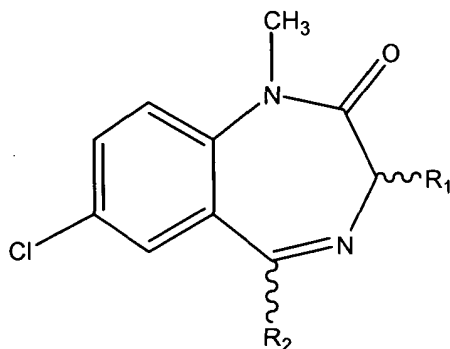
10 proliferating target cells having mitochondria, and a composition comprising the following formula:



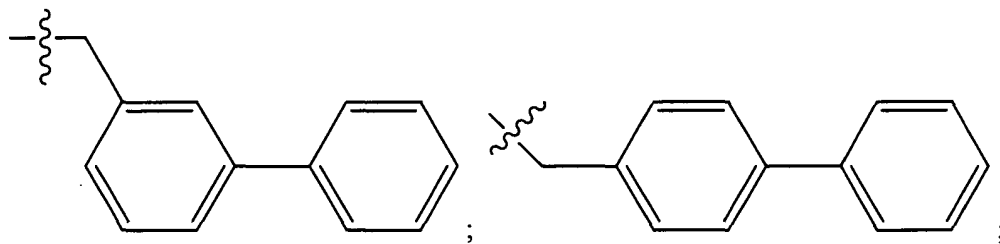
wherein R₁ comprises a hydrophobic aromatic group larger than benzene; wherein R₂ comprises a phenolic hydroxyl group; wherein R₁ and R₂ include both R or S enantiomeric forms and racemic mixtures; and wherein the cells are exposed to the composition under conditions such that the composition binds to the mitochondrial ATP synthase complex so as to increase superoxide levels or alter cellular ATP levels in the cells. In preferred embodiments, the composition binds to oligomycin sensitivity conferring protein.

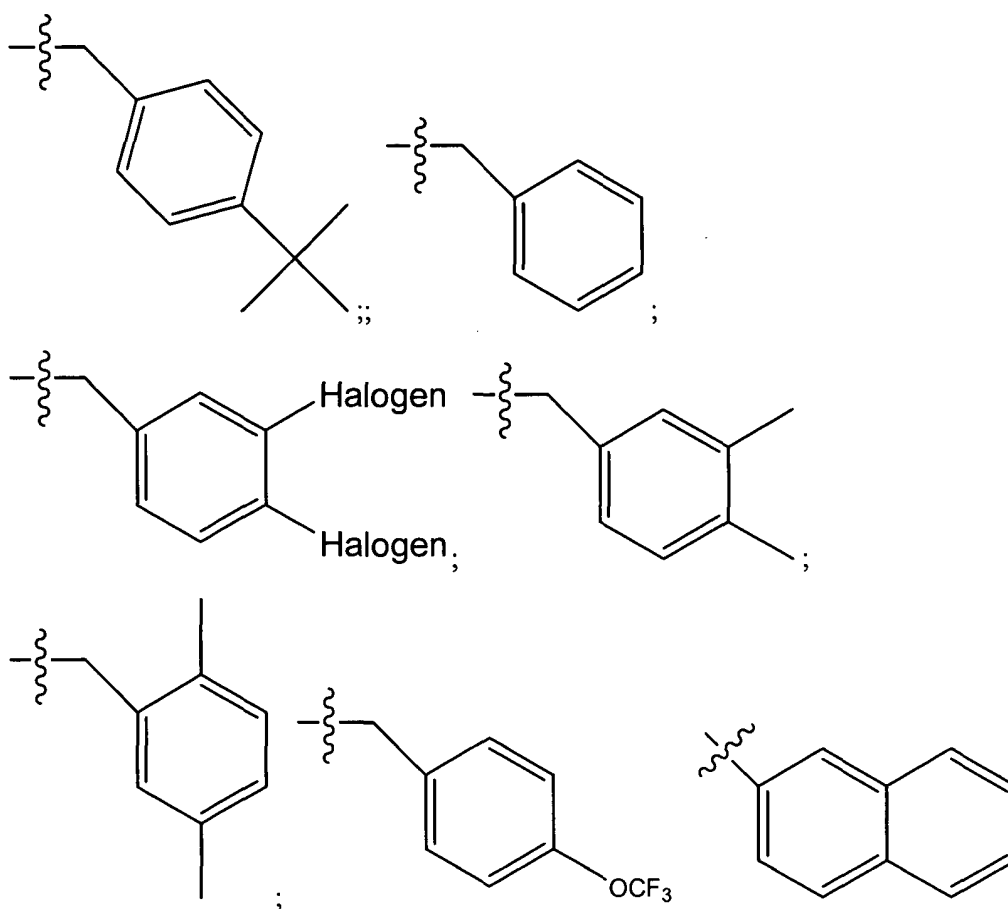
In some embodiments, the target cells are *in vitro* cells. In other embodiments, the target cells are *in vivo* cells. In still other embodiments, the target cells are *ex vivo* cells. In other embodiments, the target cells are cancer cells. In yet other embodiments, the target cells are selected from the group consisting of B cells, T cells, and granulocytes. In still further embodiments, the target cells are proliferating cells.

In other embodiments wherein the present invention regulates cellular proliferation, the present invention provides the following composition:

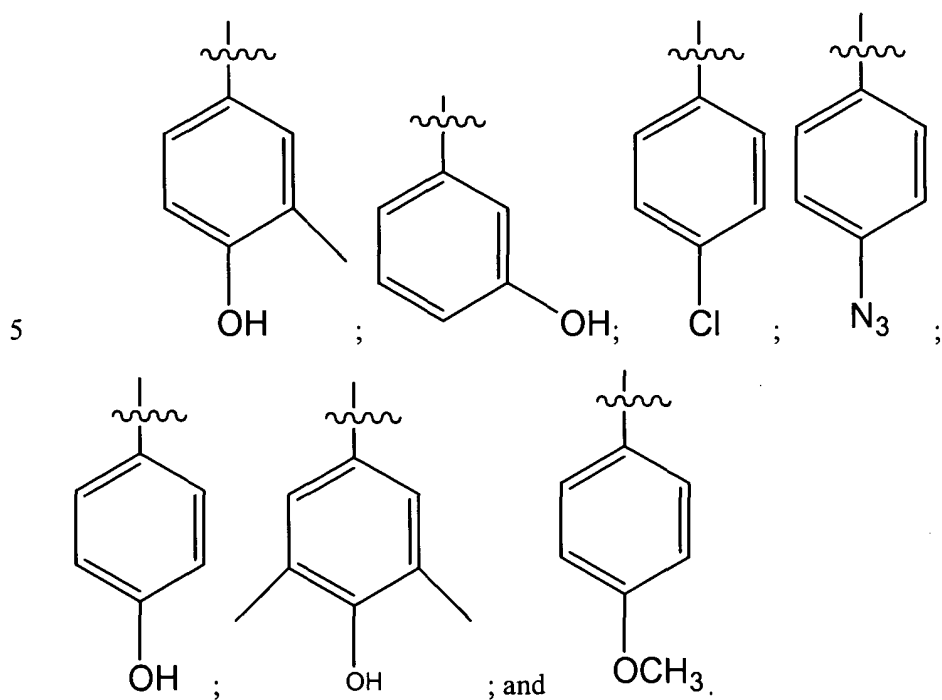


wherein R₁ is selected from naphthalalanine; phenol; 1-Napthalenol; 2-Napthalenol;

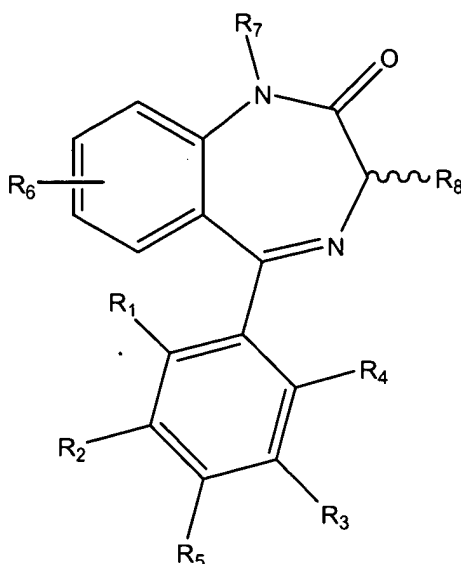




and quinolines; wherein R_2 is selected from the group consisting of:



Still other preferred embodiments of the present invention involve compositions comprising the following formula (including R and S enantiomers and racemic mixtures):



- 5 wherein R1, R2, R3 and R4 are selected from the group consisting of: hydrogen; CH₃; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one hydroxy subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one thiol subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, wherein said aliphatic chain terminates with an aldehyde subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one ketone subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons; wherein said aliphatic chain terminates with a carboxylic acid subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one amide subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one acyl group; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one nitrogen containing moiety (e.g., nitro, nitrile, etc.); a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one amine subgroup; a linear or
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- 15
- 20

branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 having at least one ether subgroup; a linear or branched, saturated or unsaturated
 aliphatic chain having at least 2 carbons, and having at least one halogen subgroup; a
 linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons,
 5 and having at least one nitronium subgroup; wherein R5 is selected from the group
 consisting of: OH; NO₂; NR'; OR'; wherein R' is selected from the group consisting
 of: a linear or branched, saturated or unsaturated aliphatic chain having at least one
 carbon; a linear or branched, saturated or unsaturated aliphatic chain having at least 2
 carbons, and having at least one hydroxyl subgroup; a linear or branched, saturated or
 10 unsaturated aliphatic chain having at least 2 carbons, and having at least one thiol
 subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least
 2 carbons, wherein said aliphatic chain terminates with an aldehyde subgroup; a linear
 or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 having at least one ketone subgroup; a linear or branched, saturated or unsaturated
 15 aliphatic chain having at least 2 carbons; wherein said aliphatic chain terminates with
 a carboxylic acid subgroup; a linear or branched, saturated or unsaturated aliphatic
 chain having at least 2 carbons, and having at least one amide subgroup; a linear or
 branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 having at least one acyl group; a linear or branched, saturated or unsaturated aliphatic
 20 chain having at least 2 carbons, and having at least one nitrogen containing moiety
 (e.g., nitro, nitrile, etc.); a linear or branched, saturated or unsaturated aliphatic chain
 having at least 2 carbons, and having at least one amine subgroup; a linear or
 branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 having at least one halogen subgroup; a linear or branched, saturated or unsaturated
 25 aliphatic chain having at least 2 carbons, and having at least one nitronium subgroup;
 wherein R6 is selected from the group consisting of: Hydrogen; NO₂; Cl; F; Br; I;
 SR'; and NR'₂, wherein R' is defined as above in R5; wherein R7 is selected from the
 group consisting of: Hydrogen; a linear or branched, saturated or unsaturated
 aliphatic chain having at least 2 carbons; and wherein R8 is an aliphatic cyclic group
 30 larger than benzene; wherein said larger than benzene comprises any chemical group
 containing 7 or more non-hydrogen atoms, and is an aryl or aliphatic cyclic group. In
 some embodiments, R' is any functional group that protects the oxygen of R5 from

metabolism in vivo, until the compound reaches its biological target (e.g., mitochondria). In some embodiments, R' protecting group(s) is metabolized at the target site, converting R5 to a hydroxyl group.

Additionally, in preferred embodiments R5 functions in interacting with
5 cellular mitochondria (i.e., in the absence of R5, the compound has reduced binding affinity for a mitochondrial component). In further embodiments, R1-R4 function to prevent undesired metabolism of the composition, and in particular a hydroxyl group at R5. In yet other embodiments, R1-R4 function to promote cellular mitochondrial metabolism of the composition. In other preferred embodiments, the interacting of
10 the composition with cellular mitochondria comprises binding the OSCP. In even further embodiments, the binding of the OSCP causes an increase in superoxide levels. In other preferred embodiments, R5 functions in regulating cellular proliferation and regulation cellular apoptosis.

The present invention also provides compositions and methods for treating
15 compromised vessels. For example, the present invention provides compositions and methods for treating compromised cardiac vessels. In preferred embodiments, the compromised vessel is an occluded vessel. In some embodiments, the present invention provides a method of treating a compromised vessel, comprising the providing of drug-eluting stent media. In preferred embodiments, the drug-eluting
20 stent media comprises a pharmaceutical composition of the present invention. In preferred embodiments, the pharmaceutical composition is coated onto the drug-eluting stent media. In further embodiments, the pharmaceutical composition comprises an agent and a pharmaceutically acceptable excipient. In preferred embodiments, the agent comprises any of the structures described herein.

25 Within the compositions and methods for treating compromised vessels in a subject suffering from a compromised vessel, the present invention further involves treating said subject with drug-eluting stent media and applying the pharmaceutical composition onto the compromised vessel. In some embodiments, the application of the pharmaceutical composition onto said compromised vessel inhibits restenosis. In
30 yet further embodiments, the application of the pharmaceutical composition inhibits smooth muscle cell differentiation, migration and proliferation.

In other embodiments, the pharmaceutical composition further comprises an adhesive agent. In some embodiments, the adhesive agent is biodegradable. In even further embodiments, the adhesive agent is fibrin glue.

5 DESCRIPTION OF THE FIGURES

Figure 1 shows data demonstrating that the OSCP component is a binding protein for Bz-423.

Figure 2 is a graph showing the binding isotherm of Bz-423 and purified human OSCP.

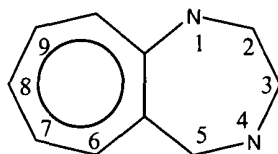
10 Figure 3 shows siRNA regulation of OSCP.

Figure 4 shows data showing gene expression profiles of cells treated by the compounds of the present invention. Data from an expression analysis for genes up-regulated in the presence of Bz-423 is presented in Figure 4A. Data from an expression analysis for genes down-regulated in the presence of Bz-423 is presented
15 in Figure 4B. Data from an expression analysis for genes up-regulated in the presence of Bz-OMe is presented in Figure 4C. Data from an expression analysis for genes down-regulated in the presence of Bz-OMe is presented in Figure 4D.

DEFINITIONS

20 To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

As used herein, the term “benzodiazepine” refers to a seven membered non-aromatic heterocyclic ring fused to a phenyl ring wherein the seven-membered ring has two nitrogen atoms, as part of the heterocyclic ring. In some aspects, the two
25 nitrogen atoms are in 1 and 4 positions, as shown in the general structure below.



The benzodiazepine can be substituted with one keto group (typically at the 2-position), or with two keto groups, one each at the 2- and 5- positions. When the benzodiazepine has two keto groups, one each at the 2- and 5- positions, it is referred
30 to as benzodiazepine-2,5-dione. Most generally, the benzodiazepine is further

substituted either on the six-membered phenyl ring or on the seven-membered heterocyclic ring or on both rings by a variety of substituents. These substituents are described more fully herein.

5 The term “larger than benzene” refers to any chemical group containing 7 or more non-hydrogen atoms.

As used herein, the term “substituted aliphatic” refers to an alkane possessing less than 10 carbons where at least one of the aliphatic hydrogen atoms has been replaced by a halogen, an amino, a hydroxy, a nitro, a thio, a ketone, an aldehyde, an ester, an amide, a lower aliphatic, a substituted lower aliphatic, or a ring (aryl,
10 substituted aryl, cycloaliphatic, or substituted cycloaliphatic, *etc.*). Examples of such include, but are not limited to, 1-chloroethyl and the like.

As used herein, the term “substituted aryl” refers to an aromatic ring or fused aromatic ring system consisting of no more than three fused rings at least one of which is aromatic, and where at least one of the hydrogen atoms on a ring carbon has
15 been replaced by a halogen, an amino, a hydroxy, a nitro, a thio, a ketone, an aldehyde, an ester, an amide, a lower aliphatic, a substituted lower aliphatic, or a ring (aryl, substituted aryl, cycloaliphatic, or substituted cycloaliphatic). Examples of such include, but are not limited to, hydroxyphenyl and the like.

As used herein, the term “cycloaliphatic” refers to a cycloalkane possessing
20 less than 8 carbons or a fused ring system consisting of no more than three fused cycloaliphatic rings. Examples of such include, but are not limited to, decalin and the like.

As used herein, the term “substituted cycloaliphatic” refers to a cycloalkane possessing less than 10 carbons or a fused ring system consisting of no more than
25 three fused rings, and where at least one of the aliphatic hydrogen atoms has been replaced by a halogen, a nitro, a thio, an amino, a hydroxy, a ketone, an aldehyde, an ester, an amide, a lower aliphatic, a substituted lower aliphatic, or a ring (aryl, substituted aryl, cycloaliphatic, or substituted cycloaliphatic). Examples of such include, but are not limited to, 1-chlorodecalyl, bicyclo-heptanes, octanes, and
30 nonanes (*e.g.*, nonbornyl) and the like.

As used herein, the term “heterocyclic” refers to a cycloalkane and/or an aryl ring system, possessing less than 8 carbons, or a fused ring system consisting of no

more than three fused rings, where at least one of the ring carbon atoms is replaced by oxygen, nitrogen or sulfur. Examples of such include, but are not limited to, morpholino and the like.

As used herein, the term "substituted heterocyclic" refers to a cycloalkane
5 and/or an aryl ring system, possessing less than 8 carbons, or a fused ring system consisting of no more than three fused rings, where at least one of the ring carbon atoms is replaced by oxygen, nitrogen or sulfur, and where at least one of the aliphatic hydrogen atoms has been replaced by a halogen, hydroxy, a thio, nitro, an amino, a ketone, an aldehyde, an ester, an amide, a lower aliphatic, a substituted lower
10 aliphatic, or a ring (aryl, substituted aryl, cycloaliphatic, or substituted cycloaliphatic). Examples of such include, but are not limited to 2-chloropyranyl.

As used herein, the term "linker" refers to a chain containing up to and including eight contiguous atoms connecting two different structural moieties where such atoms are, for example, carbon, nitrogen, oxygen, or sulfur. Ethylene glycol is
15 one non-limiting example.

As used herein, the term "lower-alkyl-substituted-amino" refers to any alkyl unit containing up to and including eight carbon atoms where one of the aliphatic hydrogen atoms is replaced by an amino group. Examples of such include, but are not limited to, ethylamino and the like.

20 As used herein, the term "lower-alkyl-substituted-halogen" refers to any alkyl chain containing up to and including eight carbon atoms where one of the aliphatic hydrogen atoms is replaced by a halogen. Examples of such include, but are not limited to, chlorethyl and the like.

As used herein, the term "acetylamino" shall mean any primary or secondary
25 amino that is acetylated. Examples of such include, but are not limited to, acetamide and the like.

The term "derivative" of a compound, as used herein, refers to a chemically modified compound wherein the chemical modification takes place either at a functional group of the compound or on the aromatic ring. Non-limiting examples of
30 1,4-benzodiazepine derivatives of the present invention may include N-acetyl, N-methyl, N-hydroxy groups at any of the available nitrogens in the compound.

Additional derivatives may include those having a trifluoromethyl group on the phenyl ring.

The term "stent" or "drug-eluting stent," as used herein, refers to any device which when placed into contact with a site in the wall of a lumen to be treated, will also place fibrin at the lumen wall and retain it at the lumen wall. This can include especially devices delivered percutaneously to treat coronary artery occlusions and to seal dissections or aneurysms of splenic, carotid, iliac and popliteal vessels. The stent can also have underlying polymeric or metallic structural elements onto which the fibrin is applied or the stent can be a composite of fibrin intermixed with a polymer. For example, a deformable metal wire stent such as that disclosed in U.S. Pat. No.: 4,886,062, herein incorporated by reference, could be coated with fibrin as set forth above in one or more coats (i.e., polymerization of fibrin on the metal framework by application of a fibrinogen solution and a solution of a fibrinogen-coagulating protein) or provided with an attached fibrin preform such as an encircling film of fibrin. The stent and fibrin could then be placed onto the balloon at a distal end of a balloon catheter and delivered by conventional percutaneous means (e.g. as in an angioplasty procedure) to the site of the restriction or closure to be treated where it would then be expanded into contact with the body lumen by inflating the balloon. The catheter can then be withdrawn, leaving the fibrin stent of the present invention in place at the treatment site. The stent may therefore provide both a supporting structure for the lumen at the site of treatment and also a structure supporting the secure placement of fibrin at the lumen wall. Generally, a drug-eluting stent allows for an active release of a particular drug at the stent implementation site.

As used herein, the term "catheter" refers generally to a tube used for gaining access to a body cavity or blood vessel.

As used herein, the term "valve" or "vessel" refers to any lumen within a mammal. Examples include, but are not limited to, arteries, veins, capillaries, and biological lumen.

As used herein, the term "restenosis" refers to any valve which is narrowed. Examples include, but are not limited to, the reclosure of a peripheral or coronary artery following trauma to that artery caused by efforts to open a stenosed portion of

the artery, such as, for example, by balloon dilation, ablation, atherectomy or laser treatment of the artery.

As used herein, “angioplasty” or “balloon therapy” or “balloon angioplasty” or “percutaneous transluminal coronary angioplasty” refers to a method of treating blood vessel disorders that involves the use of a balloon catheter to enlarge the blood vessel and thereby improve blood flow.

As used herein, “cardiac catheterization” or “coronary angiogram” refers to a test used to diagnose coronary artery disease using a catheterization procedure. Such a procedure may involve, for example, the injection of a contrast dye into the coronary arteries via a catheter, permitting the visualization of a narrowed or blocked artery.

As used herein, the term “subject” refers to organisms to be treated by the methods of the present invention. Such organisms preferably include, but are not limited to, mammals (*e.g.*, murines, simians, equines, bovines, porcines, canines, felines, and the like), and most preferably includes humans. In the context of the invention, the term “subject” generally refers to an individual who will receive or who has received treatment (*e.g.*, administration of benzodiazepine compound(s), and optionally one or more other agents) for a condition characterized by the dysregulation of apoptotic processes.

The term “diagnosed,” as used herein, refers to the recognition of a disease by its signs and symptoms (*e.g.*, resistance to conventional therapies), or genetic analysis, pathological analysis, histological analysis, and the like.

As used herein, the terms “anticancer agent,” or “conventional anticancer agent” refer to any chemotherapeutic compounds, radiation therapies, or surgical interventions, used in the treatment of cancer.

As used herein the term, “*in vitro*” refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments include, but are not limited to, test tubes and cell cultures. The term “*in vivo*” refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reaction that occur within a natural environment.

As used herein, the term “host cell” refers to any eukaryotic or prokaryotic cell (e.g., mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located *in vitro* or *in vivo*.

As used herein, the term “cell culture” refers to any *in vitro* culture of cells.

- 5 Included within this term are continuous cell lines (e.g., with an immortal phenotype), primary cell cultures, finite cell lines (e.g., non-transformed cells), and any other cell population maintained *in vitro*, including oocytes and embryos.

In preferred embodiments, the “target cells” of the compositions and methods of the present invention include, refer to, but are not limited to, lymphoid cells or
10 cancer cells. Lymphoid cells include B cells, T cells, and granulocytes. Granulocytes include eosinophils and macrophages. In some embodiments, target cells are continuously cultured cells or uncultured cells obtained from patient biopsies.

Cancer cells include tumor cells, neoplastic cells, malignant cells, metastatic cells, and hyperplastic cells. Neoplastic cells can be benign or malignant. Neoplastic
15 cells are benign if they do not invade or metastasize. A malignant cell is one that is able to invade and/or metastasize. Hyperplasia is a pathologic accumulation of cells in a tissue or organ, without significant alteration in structure or function.

In one specific embodiment, the target cells exhibit pathological growth or proliferation. As used herein, the term “pathologically proliferating or growing cells”
20 refers to a localized population of proliferating cells in an animal that is not governed by the usual limitations of normal growth.

As used herein, the term “un-activated target cell” refers to a cell that is either in the G₀ phase or one in which a stimulus has not been applied.

As used herein, the term “activated target lymphoid cell” refers to a lymphoid
25 cell that has been primed with an appropriate stimulus to cause a signal transduction cascade, or alternatively, a lymphoid cell that is not in G₀ phase. Activated lymphoid cells may proliferate, undergo activation induced cell death, or produce one or more of cytotoxins, cytokines, and other related membrane-associated proteins characteristic of the cell type (e.g., CD8⁺ or CD4⁺). They are also capable of
30 recognizing and binding any target cell that displays a particular antigen on its surface, and subsequently releasing its effector molecules.

As used herein, the term “activated cancer cell” refers to a cancer cell that has been primed with an appropriate stimulus to cause a signal transduction. An activated cancer cell may or may not be in the G₀ phase.

5 An activating agent is a stimulus that upon interaction with a target cell results in a signal transduction cascade. Examples of activating stimuli include, but are not limited to, small molecules, radiant energy, and molecules that bind to cell activation cell surface receptors. Responses induced by activation stimuli can be characterized by changes in, among others, intracellular Ca²⁺, superoxide, or hydroxyl radical levels; the activity of enzymes like kinases or phosphatases; or the energy state of the
10 cell. For cancer cells, activating agents also include transforming oncogenes.

In one aspect, the activating agent is any agent that binds to a cell surface activation receptor. These can be selected from the group consisting of a T cell receptor ligand, a B cell activating factor (“BAFF”), a TNF, a Fas ligand (FasL), a CD40 ligand, a proliferation inducing ligand (“APRIL”), a cytokine, a chemokine, a hormone, an amino
15 acid (*e.g.*, glutamate), a steroid, a B cell receptor ligand, gamma irradiation, UV irradiation, an agent or condition that enhances cell stress, or an antibody that specifically recognizes and binds a cell surface activation receptor (*e.g.*, anti-CD4, anti-CD8, anti-CD20, anti-TACI, anti-BCMA, anti-TNF receptor, anti-CD40, anti-CD3, anti-CD28, anti-B220, anti-CD38, and-CD19, and anti-CD21). BCMA is B cell maturation
20 antigen receptor and TACI is transmembrane activator and CAML interactor. (Gross, A. *et al.* (2000); Laabi, Y. *et al.* (1992) and Madry, C. *et al.* (1998)). Antibodies include monoclonal or polyclonal or a mixture thereof.

Examples of a T cell ligand include, but are not limited to, a peptide that binds to an MHC molecule, a peptide MHC complex, or an antibody that recognizes components
25 of the T cell receptor.

Examples of a B cell ligand include, but are not limited to, a molecule or antibody that binds to or recognizes components of the B cell receptor.

Examples of reagents that bind to a cell surface activation receptor include, but are not limited to, the natural ligands of these receptors or antibodies raised against them
30 (*e.g.*, anti-CD20). RITUXIN (Genentech, Inc., San Francisco, CA) is a commercially available anti-CD 20 chimeric monoclonal antibody.

Examples of agents or conditions that enhance cell stress include heat, radiation, oxidative stress, or growth factor withdrawal and the like. Examples of growth factors include, but are not limited to serum, IL-2, platelet derived growth factor ("PDGF"), and the like.

5 As used herein, the term "effective amount" refers to the amount of a compound (*e.g.*, benzodiazepine) sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages and is not limited intended to be limited to a particular formulation or administration route.

10 As used herein, the term "dysregulation of the process of cell death" refers to any aberration in the ability of (*e.g.*, predisposition) a cell to undergo cell death via either necrosis or apoptosis. Dysregulation of cell death is associated with or induced by a variety of conditions, including for example, autoimmune disorders (*e.g.*, systemic lupus erythematosus, rheumatoid arthritis, graft-versus-host disease,
15 myasthenia gravis, Sjögren's syndrome, *etc.*), chronic inflammatory conditions (*e.g.*, psoriasis, asthma and Crohn's disease), hyperproliferative disorders (*e.g.*, tumors, B cell lymphomas, T cell lymphomas, *etc.*), viral infections (*e.g.*, herpes, papilloma, HIV), and other conditions such as osteoarthritis and atherosclerosis.

 It should be noted that when the dysregulation is induced by or associated with
20 a viral infection, the viral infection may or may not be detectable at the time dysregulation occurs or is observed. That is, viral-induced dysregulation can occur even after the disappearance of symptoms of viral infection.

 A "hyperproliferative disorder," as used herein refers to any condition in which a localized population of proliferating cells in an animal is not governed by the
25 usual limitations of normal growth. Examples of hyperproliferative disorders include tumors, neoplasms, lymphomas and the like. A neoplasm is said to be benign if it does not undergo, invasion or metastasis and malignant if it does either of these. A metastatic cell or tissue means that the cell can invade and destroy neighboring body structures. Hyperplasia is a form of cell proliferation involving an increase in cell
30 number in a tissue or organ, without significant alteration in structure or function. Metaplasia is a form of controlled cell growth in which one type of fully differentiated cell substitutes for another type of differentiated cell. Metaplasia can occur in

epithelial or connective tissue cells. A typical metaplasia involves a somewhat disorderly metaplastic epithelium.

The pathological growth of activated lymphoid cells often results in an autoimmune disorder or a chronic inflammatory condition. As used herein, the term “autoimmune disorder” refers to any condition in which an organism produces antibodies or immune cells which recognize the organism's own molecules, cells or tissues. Non-limiting examples of autoimmune disorders include autoimmune hemolytic anemia, autoimmune hepatitis, Berger’s disease or IgA nephropathy, Celiac Sprue, chronic fatigue syndrome, Crohn’s disease, dermatomyositis, fibromyalgia, graft versus host disease, Grave’s disease, Hashimoto’s thyroiditis, idiopathic thrombocytopenia purpura, lichen planus, multiple sclerosis, myasthenia gravis, psoriasis, rheumatic fever, rheumatic arthritis, scleroderma, Sjorgren syndrome, systemic lupus erythematosus, type 1 diabetes, ulcerative colitis, vitiligo, and the like.

As used herein, the term “chronic inflammatory condition” refers to a condition wherein the organism’s immune cells are activated. Such a condition is characterized by a persistent inflammatory response with pathologic sequelae. This state is characterized by infiltration of mononuclear cells, proliferation of fibroblasts and small blood vessels, increased connective tissue, and tissue destruction. Examples of chronic inflammatory diseases include, but are not limited to, Crohn's disease, psoriasis, chronic obstructive pulmonary disease, inflammatory bowel disease, multiple sclerosis, and asthma. Autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus can also result in a chronic inflammatory state.

As used herein, the term “co-administration” refers to the administration of at least two agent(s) (*e.g.*, benzodiazepines) or therapies to a subject. In some embodiments, the co-administration of two or more agents/therapies is concurrent. In other embodiments, a first agent/therapy is administered prior to a second agent/therapy. Those of skill in the art understand that the formulations and/or routes of administration of the various agents/therapies used may vary. The appropriate dosage for co-administration can be readily determined by one skilled in the art. In some embodiments, when agents/therapies are co-administered, the respective agents/therapies are administered at lower dosages than appropriate for their

administration alone. Thus, co-administration is especially desirable in embodiments where the co-administration of the agents/therapies lowers the requisite dosage of a known potentially harmful (*e.g.*, toxic) agent(s).

5 As used herein, the term “toxic” refers to any detrimental or harmful effects on a cell or tissue as compared to the same cell or tissue prior to the administration of the toxicant.

As used herein, the term “pharmaceutical composition” refers to the combination of an active agent with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vivo*, *in vivo* or *ex vivo*.

10 As used herein, the term “pharmaceutically acceptable carrier” refers to any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions (*e.g.*, such as an oil/water or water/oil emulsions), and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants. (*See e.g.*, Martin, Remington's
15 Pharmaceutical Sciences, 15th Ed., Mack Publ. Co., Easton, PA [1975]).

As used herein, the term “pharmaceutically acceptable salt” refers to any pharmaceutically acceptable salt (*e.g.*, acid or base) of a compound of the present invention which, upon administration to a subject, is capable of providing a compound of this invention or an active metabolite or residue thereof. As is known to
20 those of skill in the art, “salts” of the compounds of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-
25 sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts.

Examples of bases include, but are not limited to, alkali metals (*e.g.*, sodium)
30 hydroxides, alkaline earth metals (*e.g.*, magnesium), hydroxides, ammonia, and compounds of formula NW_4^+ , wherein W is C_{1-4} alkyl, and the like.

Examples of salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, 5 heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the like. Other examples of salts include anions of the compounds of the present 10 invention compounded with a suitable cation such as Na^+ , NH_4^+ , and NW_4^+ (wherein W is a C_{1-4} alkyl group), and the like.

For therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the 15 preparation or purification of a pharmaceutically acceptable compound.

As used herein, the terms "solid phase supports" or "solid supports," are used in their broadest sense to refer to a number of supports that are available and known to those of ordinary skill in the art. Solid phase supports include, but are not limited to, silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, alumina 20 gels, and the like. As used herein, "solid supports" also include synthetic antigen-presenting matrices, cells, liposomes, and the like. A suitable solid phase support may be selected on the basis of desired end use and suitability for various protocols. For example, for peptide synthesis, solid phase supports may refer to resins such as polystyrene (*e.g.*, PAM-resin obtained from Bachem, Inc., Peninsula Laboratories, *etc.*), 25 POLYHIPE) resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TENTAGEL, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Bioscience, California).

As used herein, the term "pathogen" refers a biological agent that causes a 30 disease state (*e.g.*, infection, cancer, *etc.*) in a host. "Pathogens" include, but are not limited to, viruses, bacteria, archaea, fungi, protozoans, mycoplasma, prions, and parasitic organisms.

The terms "bacteria" and "bacterium" refer to all prokaryotic organisms, including those within all of the phyla in the Kingdom Procaryotae. It is intended that the term encompass all microorganisms considered to be bacteria including Mycoplasma, Chlamydia, Actinomyces, Streptomyces, and Rickettsia. All forms of bacteria are included within this definition including cocci, bacilli, spirochetes, spheroplasts, protoplasts, *etc.* Also included within this term are prokaryotic organisms which are gram negative or gram positive. "Gram negative" and "gram positive" refer to staining patterns with the Gram-staining process which is well known in the art. (*See e.g.*, Finegold and Martin, Diagnostic Microbiology, 6th Ed., CV Mosby St. Louis, *pp.* 13-15 [1982]). "Gram positive bacteria" are bacteria which retain the primary dye used in the Gram stain, causing the stained cells to appear dark blue to purple under the microscope. "Gram negative bacteria" do not retain the primary dye used in the Gram stain, but are stained by the counterstain. Thus, gram negative bacteria appear red.

As used herein, the term "microorganism" refers to any species or type of microorganism, including but not limited to, bacteria, archaea, fungi, protozoans, mycoplasma, and parasitic organisms. The present invention contemplates that a number of microorganisms encompassed therein will also be pathogenic to a subject.

As used herein, the term "fungi" is used in reference to eukaryotic organisms such as the molds and yeasts, including dimorphic fungi.

As used herein, the term "virus" refers to minute infectious agents, which with certain exceptions, are not observable by light microscopy, lack independent metabolism, and are able to replicate only within a living host cell. The individual particles (*i.e.*, virions) typically consist of nucleic acid and a protein shell or coat; some virions also have a lipid containing membrane. The term "virus" encompasses all types of viruses, including animal, plant, phage, and other viruses.

The term "sample" as used herein is used in its broadest sense. A sample suspected of indicating a condition characterized by the dysregulation of apoptotic function may comprise a cell, tissue, or fluids, chromosomes isolated from a cell (*e.g.*, a spread of metaphase chromosomes), genomic DNA (in solution or bound to a solid support such as for Southern blot analysis), RNA (in solution or bound to a solid support such as for Northern blot analysis), cDNA (in solution or bound to a solid

support) and the like. A sample suspected of containing a protein may comprise a cell, a portion of a tissue, an extract containing one or more proteins and the like.

As used herein, the terms "purified" or "to purify" refer, to the removal of undesired components from a sample. As used herein, the term "substantially
5 purified" refers to molecules that are at least 60% free, preferably 75% free, and most preferably 90%, or more, free from other components with which they usually associated.

As used herein, the term "antigen binding protein" refers to proteins which bind to a specific antigen. "Antigen binding proteins" include, but are not limited to,
10 immunoglobulins, including polyclonal, monoclonal, chimeric, single chain, and humanized antibodies, Fab fragments, F(ab')₂ fragments, and Fab expression libraries. Various procedures known in the art are used for the production of polyclonal antibodies. For the production of antibody, various host animals can be immunized by injection with the peptide corresponding to the desired epitope including but not
15 limited to rabbits, mice, rats, sheep, goats, *etc.* In a preferred embodiment, the peptide is conjugated to an immunogenic carrier (*e.g.*, diphtheria toxoid, bovine serum albumin (BSA), or keyhole limpet hemocyanin [KLH]). Various adjuvants are used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as
20 aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies, any technique that provides for the
25 production of antibody molecules by continuous cell lines in culture may be used (*See e.g.*, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). These include, but are not limited to, the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, *Nature*, 256:495-497 [1975]), as well as the trioma technique, the human
30 B-cell hybridoma technique (*See e.g.*, Kozbor *et al.*, *Immunol. Today*, 4:72 [1983]), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et*

al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96 [1985]).

According to the invention, techniques described for the production of single chain antibodies (U.S. 4,946,778; herein incorporated by reference) can be adapted to
5 produce specific single chain antibodies as desired. An additional embodiment of the invention utilizes the techniques known in the art for the construction of Fab expression libraries (Huse *et al.*, Science, 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibody fragments that contain the idiotype (antigen binding region) of the
10 antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragment that can be produced by pepsin digestion of an antibody molecule; the Fab' fragments that can be generated by reducing the disulfide bridges of an F(ab')₂ fragment, and the Fab fragments that can be generated by treating an antibody molecule with papain and a reducing agent.

15 Genes encoding antigen binding proteins can be isolated by methods known in the art. In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion
20 assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), Western Blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, *etc.*), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, *etc.*) *etc.*

25 As used herein, the term "immunoglobulin" or "antibody" refer to proteins that bind a specific antigen. Immunoglobulins include, but are not limited to, polyclonal, monoclonal, chimeric, and humanized antibodies, Fab fragments, F(ab')₂ fragments, and includes immunoglobulins of the following classes: IgG, IgA, IgM, IgD, IgE, and secreted immunoglobulins (sIg). Immunoglobulins generally comprise two identical
30 heavy chains and two light chains. However, the terms "antibody" and "immunoglobulin" also encompass single chain antibodies and two chain antibodies.

The term "epitope" as used herein refers to that portion of an antigen that makes contact with a particular immunoglobulin. When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as "antigenic determinants". An antigenic determinant may compete with the intact antigen (*i.e.*, the "immunogen" used to elicit the immune response) for binding to an antibody.

The terms "specific binding" or "specifically binding" when used in reference to the interaction of an antibody and a protein or peptide means that the interaction is dependent upon the presence of a particular structure (*i.e.*, the antigenic determinant or epitope) on the protein; in other words the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A," the presence of a protein containing epitope A (or free, unlabelled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

As used herein, the terms "non-specific binding" and "background binding" when used in reference to the interaction of an antibody and a protein or peptide refer to an interaction that is not dependent on the presence of a particular structure (*i.e.*, the antibody is binding to proteins in general rather than a particular structure such as an epitope).

As used herein, the term "modulate" refers to the activity of a compound (*e.g.*, benzodiazepine compound) to affect (*e.g.*, to promote or retard) an aspect of cellular function, including, but not limited to, cell growth, proliferation, apoptosis, and the like.

As used herein, the term "competes for binding" is used in reference to a first molecule (*e.g.*, a first benzodiazepine derivative) with an activity that binds to the same substrate (*e.g.*, the oligomycin sensitivity conferring protein in mitochondrial ATP synthase) as does a second molecule (*e.g.*, a second benzodiazepine derivative or other molecule that binds to the oligomycin sensitivity conferring protein in mitochondrial ATP synthase, *etc.*). The efficiency (*e.g.*, kinetics or thermodynamics) of binding by the first molecule may be the same as, or greater than, or less than, the

efficiency of the substrate binding to the second molecule. For example, the equilibrium binding constant (K_D) for binding to the substrate may be different for the two molecules.

As used herein, the term "instructions for administering said
5 compound to a subject," and grammatical equivalents thereof, includes instructions for using the compositions contained in a kit for the treatment of conditions characterized by the dysregulation of apoptotic processes in a cell or tissue (*e.g.*, providing dosing, route of administration, decision trees for treating physicians for correlating patient-specific characteristics with therapeutic courses of action). The
10 term also specifically refers to instructions for using the compositions contained in the kit to treat autoimmune disorders (*e.g.*, systemic lupus erythematosus, rheumatoid arthritis, graft-versus-host disease, myasthenia gravis, Sjögren's syndrome, *etc.*), chronic inflammatory conditions (*e.g.*, psoriasis, asthma and Crohn's disease), hyperproliferative disorders (*e.g.*, tumors, B cell lymphomas, T cell lymphomas, *etc.*),
15 viral infections (*e.g.*, herpes virus, papilloma virus, HIV), and other conditions such as osteoarthritis and atherosclerosis, and the like.

The term "test compound" refers to any chemical entity, pharmaceutical, drug, and the like, that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function, or otherwise alter the physiological or cellular status of a sample
20 (*e.g.*, the level of dysregulation of apoptosis in a cell or tissue). Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (*e.g.*, through animal trials or prior experience with administration to humans)
25 to be effective in such treatment or prevention. In preferred embodiments, "test compounds" are agents that modulate apoptosis in cells.

As used herein, the term "third party" refers to any entity engaged in selling, warehousing, distributing, or offering for sale a test compound contemplated for administered with a compound for treating conditions characterized by the
30 dysregulation of apoptotic processes.

GENERAL DESCRIPTION OF THE INVENTION

As a class of drugs, benzodiazepine compounds have been widely studied and reported to be effective medicaments for treating a number of disease. For example,
5 U.S. 4,076,823, 4,110,337, 4,495,101, 4,751,223 and 5,776,946, each incorporated herein by reference in its entirety, report that certain benzodiazepine compounds are effective as analgesic and anti-inflammatory agents. Similarly, U.S. 5,324,726 and U.S. 5,597,915, each incorporated by reference in its entirety, report that certain benzodiazepine compounds are antagonists of cholecystokinin and gastrin and thus
10 might be useful to treat certain gastrointestinal disorders.

Other benzodiazepine compounds have been studied as inhibitors of human neutrophil elastase in the treating of human neutrophil elastase-mediated conditions such as myocardial ischemia, septic shock syndrome, among others (*See e.g.*, U.S. 5,861,380 incorporated herein by reference in its entirety). U.S. 5,041,438,
15 incorporated herein by reference in its entirety, reports that certain benzodiazepine compounds are useful as anti-retroviral agents.

Despite the attention benzodiazepine compounds have drawn, it will become apparent from the description below, that the present invention provides novel benzodiazepine compounds and related compounds and methods of using the novel
20 compounds, as well as known compounds, for treating a variety of diseases.

Benzodiazepine compounds are known to bind to benzodiazepine receptors in the central nervous system (CNS) and thus have been used to treat various CNS disorders including anxiety and epilepsy. Peripheral benzodiazepine receptors have also been identified, which receptors may incidentally also be present in the CNS.
25 The present invention demonstrates that benzodiazepines and related compounds have pro-apoptotic and cytotoxic properties useful in the treatment of transformed cells grown in tissue culture. The route of action of these compounds is not through the previously identified benzodiazepine receptors.

Experiments conducted during the development of the present invention have
30 identified novel biological targets for benzodiazepine compounds and related compounds (some of which are related by their ability to bind cellular target molecules rather than their homology to the overall chemical structure of

benzodiazepine compounds). In particular, the present invention provides compounds that interact, directly or indirectly, with particular mitochondrial proteins to elicit the desired biological effects.

Thus, in some embodiments, the present invention provides a number of novel
5 compounds and previously known compounds directed against novel cellular targets to achieve desired biological results. In other embodiments, the present invention provides methods for using such compounds to regulate biological processes. The present invention also provides drug-screening methods to identify and optimize compounds. The present invention further provides diagnostic markers for
10 identifying diseases and conditions, for monitoring treatment regimens, and/or for identifying optimal therapeutic courses of action. These and other research and therapeutic utilities are described below.

DETAILED DESCRIPTION OF THE INVENTION

15 The present invention provides novel chemical compounds, methods for their discovery, and their therapeutic use. In particular, the present invention provides benzodiazepine derivatives and related compounds and methods of using benzodiazepine derivatives and related compounds as therapeutic agents to treat a number of conditions associated with the faulty regulation of the processes of
20 programmed cell death, autoimmunity, inflammation, and hyperproliferation, and the like.

Exemplary compositions and methods of the present invention are described in more detail in the following sections: I. Modulators of Cell Death; II. Modulators of Cell Growth and Proliferation; III. Expression Analysis of Treated Cells; IV.
25 Exemplary Compounds; V. Pharmaceutical compositions, formulations, and exemplary administration routes and dosing considerations; VI. Drug screens; and VII. Therapeutic Applications.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of organic chemistry, pharmacology, molecular biology
30 (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular cloning: a laboratory manual" Second Edition (Sambrook *et al.*, 1989); "Oligonucleotide synthesis" (M.J. Gait, ed., 1984); "Animal

cell culture" (R.I. Freshney, ed., 1987); the series "Methods in enzymology" (Academic Press, Inc.); "Handbook of experimental immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene transfer vectors for mammalian cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current protocols in molecular biology" (F.M. Ausubel *et al.*, eds., 1987, and periodic updates); "PCR: the polymerase chain reaction" (Mullis *et al.*, eds., 1994); and "Current protocols in immunology" (J.E. Coligan *et al.*, eds., 1991), each of which is herein incorporated by reference in its entirety.

I. Modulators of Cell Death

10 In preferred embodiments, the present invention regulates apoptosis through the exposure of cells to compounds. The effect of compounds can be measured by detecting any number of cellular changes. Cell death may be assayed as described herein and in the art. In preferred embodiments, cell lines are maintained under appropriate cell culturing conditions (*e.g.*, gas (CO₂), temperature and media) for an
15 appropriate period of time to attain exponential proliferation without density dependent constraints. Cell number and or viability are measured using standard techniques, such as trypan blue exclusion/hemo-cytometry, or MTT dye conversion assay. Alternatively, the cell may be analyzed for the expression of genes or gene products associated with aberrations in apoptosis or necrosis.

20 In preferred embodiments, exposing the present invention to a cell induces apoptosis. In some embodiments, the present invention causes an initial increase in cellular ROS levels (*e.g.*, O₂⁻). In further embodiments, exposure of the compounds of the present invention to a cell causes an increase in cellular O₂⁻ levels. In still further embodiments, the increase in cellular O₂⁻ levels resulting from the compounds
25 of the present invention is detectable with a redox-sensitive agent that reacts specifically with O₂⁻ (*e.g.*, dihydroethidium (DHE)).

In other embodiments, increased cellular O₂⁻ levels resulting from compounds of the present invention diminish after a period of time (*e.g.*, 10 minutes). In other
embodiments, increased cellular O₂⁻ levels resulting from the compounds of the
30 present invention diminish after a period of time and increase again at a later time (*e.g.*, 10 hours). In further embodiments, increased cellular O₂⁻ levels resulting from the compounds of the present invention diminish at 1 hour and increase again after 4

hours. In preferred embodiments, an early increase in cellular O_2^- levels, followed by a diminishing in cellular O_2^- levels, followed by another increase in cellular O_2^- levels resulting from the compounds of the present invention is due to different cellular processes (*e.g.*, bimodal cellular mechanisms).

5 In some embodiments, the present invention causes a collapse of a cell's mitochondrial $\Delta\Psi_m$. In preferred embodiments, a collapse of a cell's mitochondrial $\Delta\Psi_m$ resulting from the present invention is detectable with a mitochondria-selective potentiometric probe (*e.g.*, DiOC₆). In further embodiments, a collapse of a cell's mitochondrial $\Delta\Psi_m$ resulting from the present invention occurs after an initial increase
10 in cellular O_2^- levels.

 In some embodiments, the present invention enables caspase activation. In other embodiments, the present invention causes the release of cytochrome *c* from mitochondria. In further embodiments, the present invention alters cytosolic cytochrome *c* levels. In still other embodiments, altered cytosolic cytochrome *c* levels
15 resulting from the present invention are detectable with immunoblotting cytosolic fractions. In preferred embodiments, diminished cytosolic cytochrome *c* levels resulting from the present invention are detectable after a period of time (*e.g.*, 10 hours). In further preferred embodiments, diminished cytosolic cytochrome *c* levels resulting from the present invention are detectable after 5 hours.

20 In other embodiments, the present invention causes the opening of the mitochondrial PT pore. In preferred embodiments, the cellular release of cytochrome *c* resulting from the present invention is consistent with a collapse of mitochondrial $\Delta\Psi_m$. In still further preferred embodiments, the present invention causes an increase in cellular O_2^- levels after a mitochondrial $\Delta\Psi_m$ collapse and a release of cytochrome
25 *c*. In further preferred embodiments, a rise in cellular O_2^- levels is caused by a mitochondrial $\Delta\Psi_m$ collapse and release of cytochrome *c* resulting from the present invention.

 In other embodiments, the present invention causes cellular caspase activation. In preferred embodiments, caspase activation resulting from the present invention is
30 measurable with a pan-caspase sensitive fluorescent substrate (*e.g.*, FAM-VAD-fmk). In still further embodiments, caspase activation resulting from the present invention tracks with a collapse of mitochondrial $\Delta\Psi_m$. In other embodiments, the present

invention causes an appearance of hypodiploid DNA. In preferred embodiments, an appearance of hypodiploid DNA resulting from the present invention is slightly delayed with respect to caspase activation.

5 In some embodiments, the molecular target for the present invention is found within mitochondria. In further embodiments, the molecular target of the present invention involves the mitochondrial ATPase. The primary sources of cellular ROS include redox enzymes and the mitochondrial respiratory chain (hereinafter MRC). In preferred embodiments, cytochrome *c* oxidase (complex IV of the MRC) inhibitors (*e.g.*, NaN₃) preclude a present invention dependent increase in cellular ROS levels.

10 In other preferred embodiments, the ubiquinol-cytochrome *c* reductase component of MRC complex III inhibitors (*e.g.*, FK506) preclude a present invention dependent increase in ROS levels.

In some embodiments, an increase in cellular ROS levels due to the compounds of the present invention result from the binding of the compounds of the present invention to a target within mitochondria. In preferred embodiments, the compounds of the present invention oxidizes 2',7'-dichlorodihydrofluorescein (hereinafter DCF) diacetate to DCF. DCF is a redox-active species capable of generating ROS. In further embodiments, the rate of DCF production resulting from the present invention increases after a lag period.

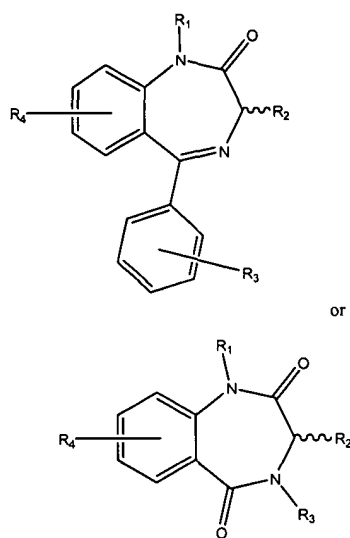
20 Antimycin A generates O₂⁻ by inhibiting ubiquinol-cytochrome *c* reductase. In preferred embodiments, the present invention increases the rate of ROS production in an equivalent manner to antimycin A. In further embodiments, the present invention increases the rate of ROS production in an equivalent manner to antimycin A under aerobic conditions supporting state 3 respiration. In further embodiments, the compounds of the present invention do not directly target the MPT pore. In additional embodiments, the compounds of the present invention do not generate substantial ROS in the subcellular S15 fraction (*e.g.*, cytosol; microsomes). In even further embodiments, the compounds of the present invention do not stimulate ROS if mitochondria are in state 4 respiration.

30 MRC complexes I – III are the primary sources of ROS within mitochondria. In preferred embodiments, the primary source of an increase in cellular ROS levels resulting from the dependent invention emanates from these complexes as a result of

inhibiting the mitochondrial F_1F_0 -ATPase. Indeed, in still further embodiments, the present invention inhibits mitochondrial ATPase activity of bovine sub-mitochondrial particles (hereinafter SMPs). In particularly preferred embodiments, the compounds of the present invention bind to the OSCP component of the mitochondrial F_1F_0 -

5 ATPase.

In some embodiments, the compounds of the present invention have the structure:



10

or its enantiomer, wherein, R_1 is aliphatic or aryl; R_2 is aliphatic, aryl, $-NH_2$, $-HC(=O)-R_5$, or a moiety that participates in hydrogen bond formation, wherein R_5 is aryl, heterocyclic, $-R_6-NH-C(=O)-R_7$ or $-R_6-C(=O)-NH-R_7$, wherein R_6 is an aliphatic linker of 1-6 carbons and R_7 is aliphatic, aryl, or heterocyclic; and each of R_3 and R_4 is independently hydrogen, hydroxy, alkoxy, halo, amino, lower-alkyl-substituted-amino, acylamino, hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, aryl, or heteroaryl; or a pharmaceutically acceptable salt, prodrug or derivative thereof. In some preferred embodiments, where R_3 is a hydroxyl group, one or more additional positions on the ring containing R_3 includes a chemical group (e.g., an alkyl chain) that protects the hydroxyl group from metabolism *in vivo*.

15

20

In certain embodiments, the compounds of the present invention may have a hydroxyl group at the C'4 position and an aromatic ring. In preferred embodiments, compounds of the present invention cause an increase in cellular ROS levels as a

result of a hydroxyl group at the C'4 position and an aromatic ring. In further embodiments, the potency of the present invention in cell based assays correlates with ATPase inhibition experiments using SMPs. Indeed, in preferred embodiments, the present invention significantly inhibits mitochondrial ATPase activity in comparison
5 to cytotoxic (80 μ M) concentrations of general benzodiazepines and PBR ligands (*e.g.*, PK11195 and 4-chlorodiazepam) that do not significantly inhibit mitochondrial ATPase activity. As such, in preferred embodiments, the molecular target of the present invention is the mitochondrial ATPase.

Oligomycin is a macrolide natural product that binds to the mitochondrial
10 F_1F_0 -ATPase, induces a state 3 to 4 transition, and as a result, generates ROS (*e.g.*, $O_2^{\cdot -}$). In preferred embodiments, the present invention binds the OSCP component of the mitochondrial F_1F_0 -ATPase. In certain embodiments, screening assays of the present invention permit detection of binding partners of the OSCP. OSCP is an intrinsically fluorescent protein. In certain embodiments, titrating a solution of test
15 compounds of the present invention into an *E. Coli* sample overexpressed with OSCP results in quenching of the intrinsic OSCP fluorescence. In other embodiments, fluorescent or radioactive test compounds can be used in direct binding assays. In other embodiments, competition binding experiments can be conducted. In this type of assay, test compounds are assessed for their ability to compete with Bz-423 for
20 binding to the OSCP. In some embodiments, the compounds of the present invention cause a reduced increase in cellular ROS levels and reduced apoptosis in cells through regulation of the OSCP gene (*e.g.*, altering expression of the OSCP gene). In further embodiments, the present invention functions by altering the molecular motions of the ATPase motor.

25 II. Modulators of Cellular Proliferation and Cell Growth

In some embodiments, the compounds and methods of the present invention cause decreased cellular proliferation. In other embodiments, the compounds and methods of the present invention causes decreased cellular proliferation and
30 apoptosis. For example, cell culture cytotoxicity assays conducted during the development of the present invention demonstrated that the compounds and methods

of the present invention prevents cell growth after an extended period in culture (*e.g.*, 3 days).

III. Expression Analysis of Treated Cells

5 In some embodiments, induced cell death is not dependent upon new protein synthesis. Treatment of cells with cyclohexamide inhibits new protein synthesis. In some embodiments, cells treated with cyclohexamide and the compounds of the present invention enter apoptosis.

 During the development of the present invention, an expression profile was
10 generated to identify those genes that are differentially expressed in treated and untreated cells. This profile provides a gene expression fingerprint of cells induced by the compounds of the present invention. This fingerprint identifies genes that are upregulated and downregulated in response to the compounds of the present invention and identifies such genes are diagnostic markers for drug screening and for
15 monitoring therapeutic effects of the compounds. The genes also provide targets for regulation to mimic the effects of the compounds of the present invention. Data from an expression analysis for genes up-regulated in the presence of Bz-423 is presented in Figure 4A. Data from an expression analysis for genes down-regulated in the presence of Bz-423 is presented in Figure 4B. Data from an expression analysis for
20 genes up-regulated in the presence of Bz-OMe is presented in Figure 4C. Data from an expression analysis for genes down-regulated in the presence of Bz-OMe is presented in Figure 4D.

 For example, an analysis of the expression profile provides ornithine decarboxylase antizyme 1 (OAZ1) as a novel therapeutic agent. OAZ1 is an
25 important regulatory protein that controls the synthesis and transport into cells of polyamines, including putrescine, spermidine and spermine. The synthesis of polyamines in cells involves several enzymatic steps, however ornithine decarboxylase is the enzyme that principally regulates this process. By inhibiting the polyamine transporter located in the plasma membrane and by targeting ornithine
30 decarboxylase for proteolytic degradation, OAZ1 reduces polyamine levels in cells. Polyamines are essential for the survival and growth of cells. Abnormal accumulation of polyamines contributes to tumor induction, cancer growth and metastasis.

Inhibitors of polyamine biosynthesis, and specifically one molecule identified as difluoromethylornithine (DFMO), are in clinical trials to confirm their anticarcinogenic and therapeutic potential. In preferred embodiments of the present invention, OAZ1 is induced to a level 16-fold above the level of control cells in cells
5 treated with the compounds of the present invention. Any method, direct or indirect, for inducing OAZ1 levels is contemplated by the present invention (*e.g.*, treatment with compounds of the present invention, gene therapy, etc.).

OAZ1 is an important regulator of polyamine metabolism and functions to decrease polyamine levels by acting as an inhibitor of ornithine decarboxylase (ODC),
10 a mitochondrial enzyme that controls the rate-limiting step of polyamine biosynthesis. After inhibition with antizyme, ODC is targeted for proteosomal degradation. Polyamines are intimately involved in cellular stability and required for cell proliferation. Inhibiting polyamine synthesis suppresses proliferation. As such, in still further embodiments, ODC expression or activity is decreased (*e.g.*, using
15 siRNA, antisense oligonucleotides, gene therapy, known or later identified inhibitors, the compounds of the present invention, etc.) to elicit the desired biological effect.

Antizyme 1 expression is regulated transcriptionally and at the post-transcriptional level. Post-transcriptional regulation plays a particularly important role in the regulation of this gene product and occurs by a unique translational
20 frameshift that depends on either polyamines (through a negative-feedback loop) or agmatine, another metabolite of arginine. ODC activity levels may be obtained by quantifying the conversion of ornithine to putrescine using ^3H -ornithine. In some embodiments, treating cells with the compounds of the present invention significantly reduces ODC activity in a dose-dependant fashion. In still further embodiments, a
25 reduction in ODC activity is paralleled by a decrease in ODC protein levels measured under similar conditions. Cells pre-incubated with MnTBAP decrease ROS levels. In some embodiments, cells pre-incubated with MnTBAP that are exposed to the compounds of the present invention display reversed inhibition of ODC.

In preferred embodiments, cells treated with high levels (*e.g.*, $>10\ \mu\text{M}$) of the
30 compounds of the present invention generate sufficient amounts of ROS that are not detoxified by cellular anti-oxidants, and result in apoptosis within a short time period (*e.g.*, 18 h). In preferred embodiments, cells treated with lower levels (*e.g.*, $<10\ \mu\text{M}$)

of the compounds of the present invention induce a reduced ROS response that is insufficient to trigger apoptosis, but is capable of inhibiting ODC or otherwise blocking cellular proliferation. In other embodiments, a derivative of the compounds of the present invention in which the phenolic hydroxyl is replaced by Cl or OCH₃ is minimally cytotoxic, generates a small ROS response in cells, binds less tightly to the OSCP, and inhibits ODC activity. In still other embodiments, cells treated with a derivative of the compounds of the present invention in which the phenolic hydroxyl is replaced by Cl experience reduced proliferation to a similar extent as to the unmodified compounds. As such, in preferred embodiments, the antiproliferative effects are obtained using chemical derivatives of the compounds of the present invention that block proliferation without inducing apoptosis.

In response to antigenic or mitogenic stimulation, lymphocytes secrete protein mediators, one of which is named migration inhibitory factor (MIF) for its ability to prevent the migration of macrophages *in vitro*. MIF may be an anti-tumor agent. In addition, the ability of MIF to prevent the migration of macrophages may be exploited for treating wounds. MIF may alter the immune response to different antigens. MIF links chemical and immunological detoxification systems. MIF was induced approximately 10-fold by Bz-423. Thus, the present invention contemplates the use of MIF as a target of the compounds of the present invention.

Profilin is induced at high levels in cell treated with the present invention. Profilin binds to actin monomers and interacts with several proteins and phosphoinositides, linking signaling pathways to the cytoskeleton. Profilin can sequester actin monomers, increase exchange of ATP for ADP on actin, and increase the rate of actin filament turnover. A comparison between several different tumorigenic cancer cell lines with nontumorigenic lines show consistently lower profilin 1 levels in tumor cells. Transfection of profilin 1 cDNA into CAL51 breast cancer cells raised the profilin 1 level, had a prominent effect on cell growth, and suppressed tumorigenicity of the overexpressing cell clones in nude mice. Therefore, induction of profilin 1 (*e.g.*, by the compounds of the present invention or otherwise) may suppress the tumorigenesis of cancer cells.

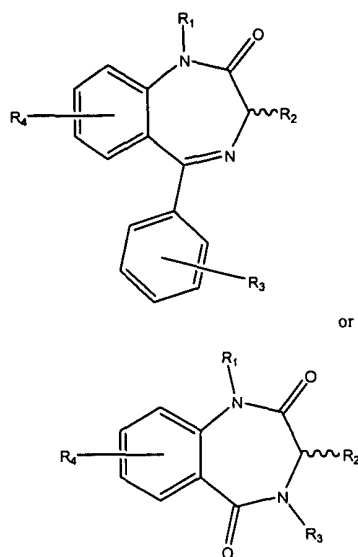
Interferon regulatory factor 4 (IRF-4) is induced at higher than normal levels in cells treated with the compounds of the present invention. IRF-4 is a

lymphoid/myeloid-restricted member of the IRF transcription factor family that plays an essential role in the homeostasis and function of mature lymphocytes. IRF-4 expression is regulated in resting primary T cells and is transiently induced at the mRNA and protein levels after activation by stimuli such as TCR cross-linking or
5 treatment with phorbol ester and calcium ionophore (PMA/ionomycin). Stable expression of IRF-4 in Jurkat cells leads to a strong enhancement in the synthesis of interleukin (IL)-2, IL-4, IL-10, and IL-13. IRF-4 represents one of the lymphoid-specific components that control the ability of T lymphocytes to produce a distinctive array of cytokines. In Abelson-transformed pro-B cell lines, enforced expression of
10 IRF-4 is sufficient to induce germline Igk transcription. The action of the compounds of the present invention to induce IRF-4 may account for its effects on autoimmune disease in B and T cell dominant processes as well as for its ability to influence the survival of neoplastic B cell clones.

In preferred embodiments, cell death-regulatory protein GRIM19 is induced at
15 higher than normal levels in cells treated with the compounds of the present invention. The importance of the interferon (IFN) pathway in cell growth suppression is known. Studies have shown that a combination of IFN and all-trans retinoic acid inhibits cell growth in vitro and in vivo more potently than either agent alone. The specific genes that play a role in IFN/RA-induced cell death were identified by an antisense
20 knockout approach, and called GRIM genes. GRIM19 is a novel cell death-associated gene that is not included in any of the known death gene categories. This gene encodes a 144-aa protein that localizes to the nucleus. Overexpression of GRIM19 enhances caspase-9 activity and apoptotic cell death in response to IFN/RA treatment. GRIM19 is located in the 19p13.2 region of the human chromosome essential for
25 prostate tumor suppression, signifying that the protein may be a novel tumor suppressor. The induction of GRIM19 by the compounds of the present invention may result in anti-tumor effects.

IV. Exemplary Compounds

30 Exemplary compounds of the present invention are provided below.



or its enantiomer, wherein, R_1 is aliphatic or aryl; R_2 is aliphatic, aryl, $-NH_2$, $-NHC(=O)-R_5$; or a moiety that participates in hydrogen bonding, wherein R_5 is aryl, heterocyclic, $-R_6-NH-C(=O)-R_7$ or $-R_6-C(=O)-NH-R_7$, wherein R_6 is an aliphatic linker of 1-6 carbons and R_7 is aliphatic, aryl, or heterocyclic, each of R_3 and R_4 is independently a hydroxy, alkoxy, halo, amino, lower-alkyl-substituted-amino, acetylamino, hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, aryl, or heterocyclic; or a pharmaceutically acceptable salt, prodrug or derivative thereof.

In the above structures, R_1 is a hydrocarbyl group of 1-20 carbons and 1-20 hydrogens. Preferably, R_1 has 1-15 carbons, and more preferably, has 1-12 carbons. Preferably, R_1 has 1-12 hydrogens, and more preferably, 1-10 hydrogens. Thus R_1 can be an aliphatic group or an aryl group.

The term "aliphatic" represents the groups commonly known as alkyl, alkenyl, alkynyl, alicyclic. The term "aryl" as used herein represents a single aromatic ring such as a phenyl ring, or two or more aromatic rings that are connected to each other (e.g., bisphenyl) or fused together (e.g., naphthalene or anthracene). The aryl group can be optionally substituted with a lower aliphatic group (e.g., C_1 - C_4 alkyl, alkenyl, alkynyl, or C_3 - C_6 alicyclic). Additionally, the aliphatic and aryl groups can be further substituted by one or more functional groups such as $-NH_2$, $-NHCOCH_3$, $-OH$, lower

alkoxy (C₁-C₄), halo (-F, -Cl, -Br, or -I). It is preferable that R₁ is primarily a nonpolar moiety.

In the above structures, R₂ can be aliphatic, aryl, -NH₂, -NHC(=O)-R₅, or a moiety that participates in hydrogen bonding, wherein R₅ is aryl, heterocyclic, R₆-NH-C(=O)-R₇ or -R₆-C(=O)-NH-R₇, wherein R₆ is an aliphatic linker of 1-6 carbons and R₇ is an aliphatic, aryl, or heterocyclic. The terms "aliphatic" and "aryl" are as defined above.

The term "a moiety that participates in hydrogen bonding" as used herein represents a group that can accept or donate a proton to form a hydrogen bond thereby.

Some specific non-limiting examples of moieties that participate in hydrogen bonding include a fluoro, oxygen-containing and nitrogen-containing groups that are well-known in the art. Some examples of oxygen-containing groups that participate in hydrogen bonding include: hydroxy, lower alkoxy, lower carbonyl, lower carboxyl, lower ethers and phenolic groups. The qualifier "lower" as used herein refers to lower aliphatic groups (C₁-C₄) to which the respective oxygen-containing functional group is attached.

Thus, for example, the term "lower carbonyl" refers to *inter alia*, formaldehyde, acetaldehyde.

Some nonlimiting examples of nitrogen-containing groups that participate in hydrogen bond formation include amino and amido groups. Additionally, groups containing both an oxygen and a nitrogen atom can also participate in hydrogen bond formation. Examples of such groups include nitro, N-hydroxy and nitrous groups.

It is also possible that the hydrogen-bond acceptor in the present invention can be the π electrons of an aromatic ring. However, the hydrogen bond participants of this invention do not include those groups containing metal atoms such as boron. Further the hydrogen bonds formed within the scope of practicing this invention do not include those formed between two hydrogens, known as "dihydrogen bonds." (See, R.H. Crabtree, Science, 282:2000-2001 [1998], for further description of such dihydrogen bonds).

The term "heterocyclic" represents, for example, a 3-6 membered aromatic or nonaromatic ring containing one or more heteroatoms. The heteroatoms can be the

same or different from each other. Preferably, at least one of the heteroatom's is nitrogen. Other heteroatoms that can be present on the heterocyclic ring include oxygen and sulfur.

Aromatic and nonaromatic heterocyclic rings are well-known in the art. Some
5 nonlimiting examples of aromatic heterocyclic rings include pyridine, pyrimidine, indole, purine, quinoline and isoquinoline. Nonlimiting examples of nonaromatic heterocyclic compounds include piperidine, piperazine, morpholine, pyrrolidine and pyrazolidine. Examples of oxygen containing heterocyclic rings include, but not limited to furan, oxirane, 2H-pyran, 4H-pyran, 2H-chromene, and benzofuran.
10 Examples of sulfur-containing heterocyclic rings include, but are not limited to, thiophene, benzothiophene, and parathiazine.

Examples of nitrogen containing rings include, but not limited to, pyrrole, pyrrolidine, pyrazole, pyrazolidine, imidazole, imidazoline, imidazolidine, pyridine, piperidine, pyrazine, piperazine, pyrimidine, indole, purine, benzimidazole, quinoline,
15 isoquinoline, triazole, and triazine.

Examples of heterocyclic rings containing two different heteroatoms include, but are not limited to, phenothiazine, morpholine, parathiazine, oxazine, oxazole, thiazine, and thiazole.

The heterocyclic ring is optionally further substituted with one or more groups
20 selected from aliphatic, nitro, acetyl (*i.e.*, -C(=O)-CH₃), or aryl groups.

Each of R₃ and R₄ can be independently a hydroxy, alkoxy, halo, amino, or substituted amino (such as lower-alkyl-substituted-amino, or acetylamino or hydroxyamino), or an aliphatic group having 1-8 carbons and 1-20 hydrogens. When
25 each of R₃ and R₄ is an aliphatic group, it can be further substituted with one or more functional groups such as a hydroxy, alkoxy, halo, amino or substituted amino groups as described above. The terms "aliphatic" is defined above. Alternatively, each of R₃ and R₄ can be hydrogen.

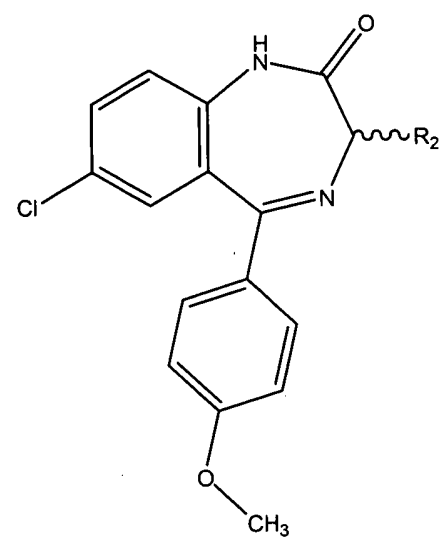
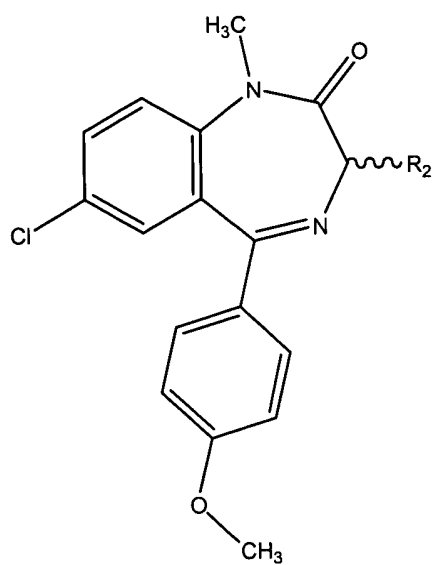
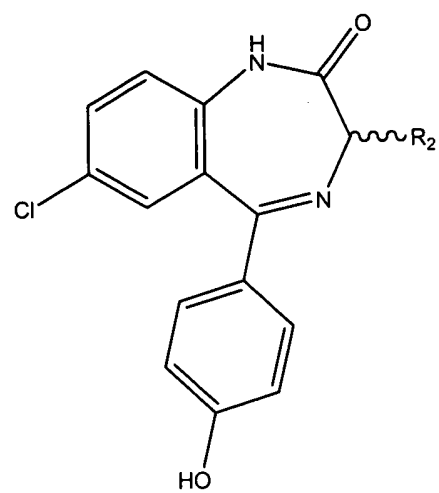
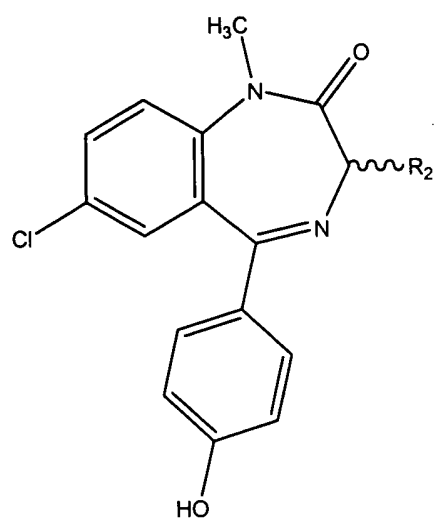
It is well-known that many 1,4-benzodiazepines exist as optical isomers due to the chirality introduced into the heterocyclic ring at tile C₃ position. The optical
30 isomers are sometimes described as L- or D-isomers in the literature. Alternatively, the isomers are also referred to as R- and S- enantiomorphs. For the sake of simplicity, these isomers are referred to as enantiomorphs or enantiomers. The 1,4-

benzodiazepine compounds described herein include their enantiomeric forms as well as racemic mixtures. Thus, the usage "benzodiazepine or its enantiomers" herein refers to the benzodiazepine as described or depicted, including all its enantiomorphs as well as their racemic mixture.

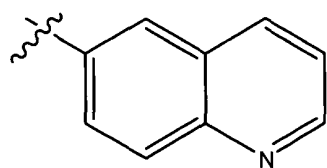
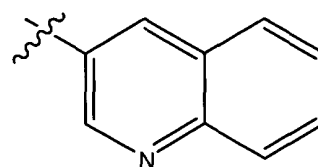
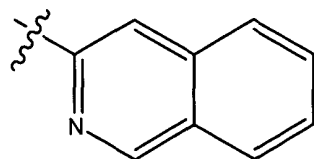
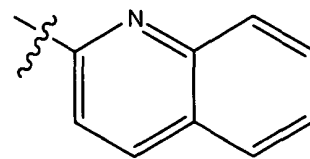
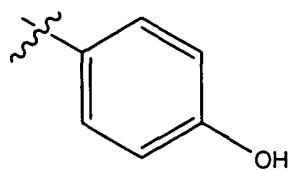
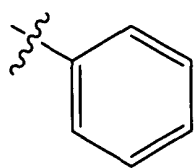
5 From the above description, it is apparent that many specific examples are represented by the generic formulas presented above. Thus, in one example, R₁ is aliphatic, R₂ is aliphatic, whereas in another example, R₁ is aryl and R₂ is a moiety that participates in hydrogen bond formation. Alternatively, R₁ can be aliphatic, and R₂ can be an -NHC(=O)-R₅, or a moiety that participates in hydrogen bonding,
10 wherein R₅ is aryl, heterocyclic, -R₆-NH-C(=O)-R₇ or -R₆-C(=O)-NH-R₇, wherein R₆ is an aliphatic linker of 1-6 carbons and R₇ is an aliphatic, aryl, or heterocyclic. A wide variety of sub combinations arising from selecting a particular group at each substituent position are possible and all such combinations are within the scope of this invention.

15 Further, it should be understood that the numerical ranges given throughout this disclosure should be construed as a flexible range that contemplates any possible subrange within that range. For example, the description of a group having the range of 1-10 carbons would also contemplate a group possessing a subrange of, for example, 1-3, 1-5, 1-8, or 2-3, 2-5, 2-8, 3-4, 3-5, 3-7, 3-9, 3-10, *etc.*, carbons. Thus,
20 the range 1-10 should be understood to represent the outer boundaries of the range within which many possible subranges are clearly contemplated. Additional examples contemplating ranges in other contexts can be found throughout this disclosure wherein such ranges include analogous subranges within.

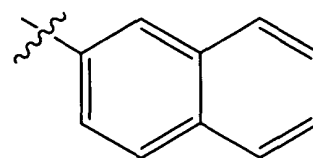
 Some specific examples of the benzodiazepine compounds of this invention
25 include:



wherein R₂ is



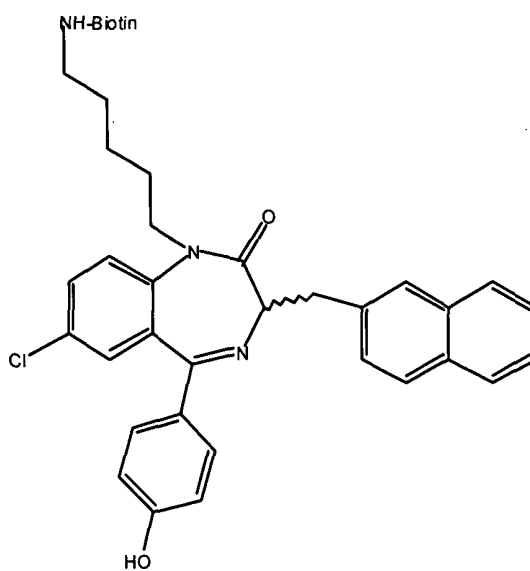
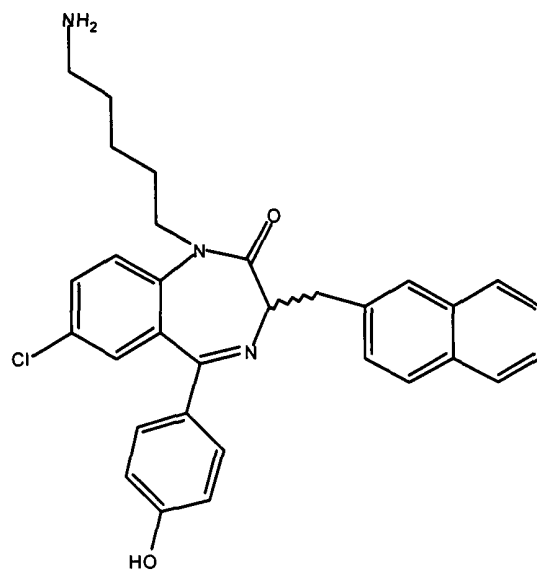
and



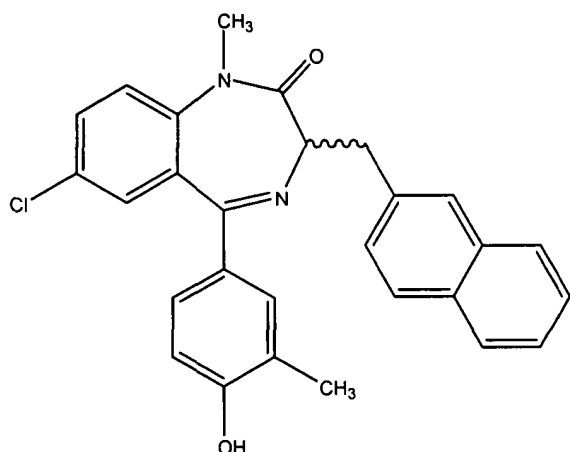
and dimethylphenyl (all isomers) and ditrifluoromethyl (all isomers).

5

The following compounds are also contemplated:



This invention also provides the compound Bz-423.

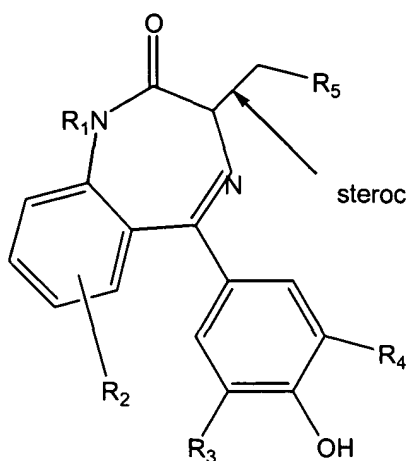


5 Bz-423 differs from benzodiazepines in clinical use by the presence of a hydrophobic substituent at C-3. This substitution renders binding to the peripheral benzodiazepine receptor ("PBR") weak (K_d ca. 1 μ M) and prevents binding to the central benzodiazepine receptor so that Bz-423 is not a sedative.

10 In some embodiments R2 is any chemical group that permits the compound to bind to OSCP. In some such embodiments, R2 comprises a hydrophobic aromatic group. In preferred embodiments R2 comprises a hydrophobic aromatic group larger than benzene (*e.g.*, a benzene ring with non-hydrogen substituents, a moiety having two or more aromatic rings, a moiety with 7 or more carbon atoms, etc.).

Additional specific benzodiazepine derivative examples of the present invention include the following:

15



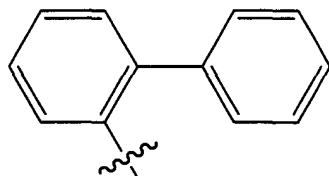
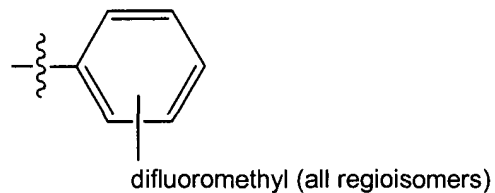
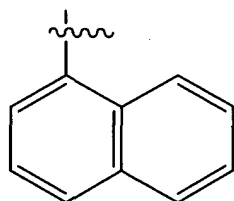
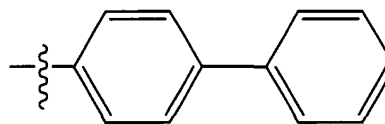
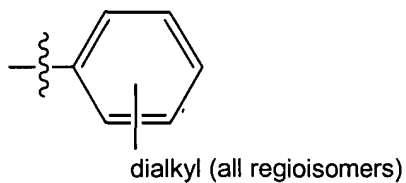
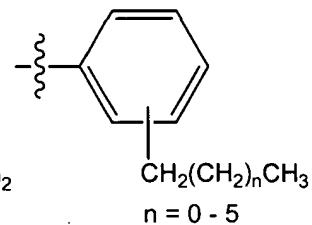
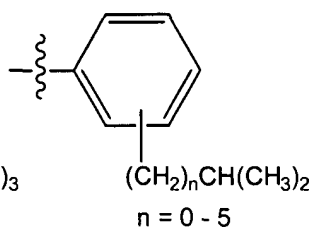
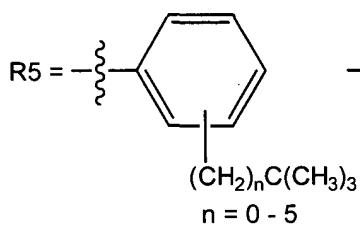
R1 = H, alkyl, or substituted alkyl

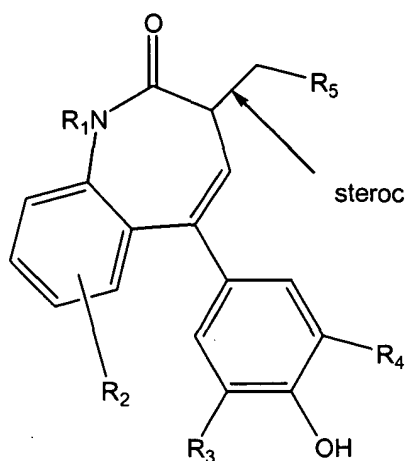
R3 = H, alkyl, or substituted alkyl

R4 = H, alkyl, or substituted alkyl

stereochemistry is R, S, or racemic

R2 is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic

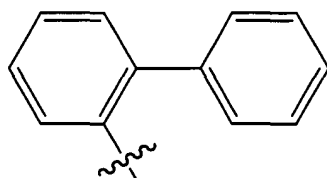
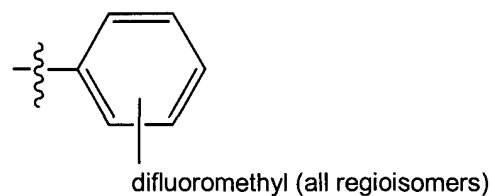
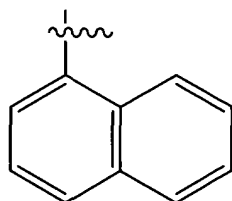
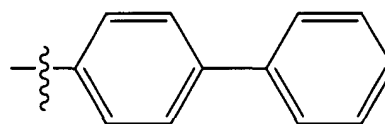
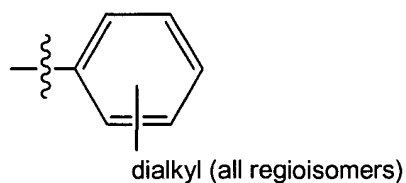
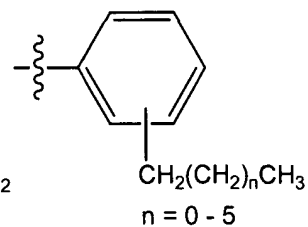
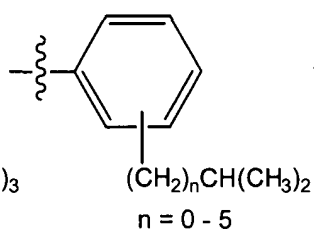
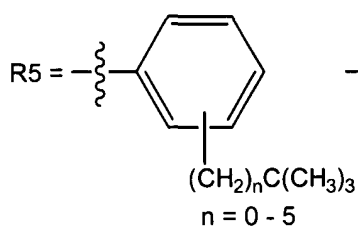


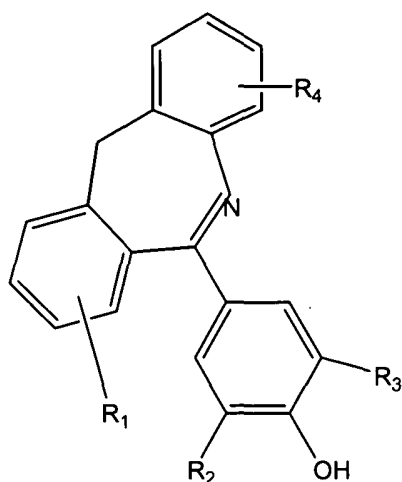


R1 = H, alkyl, or substituted alkyl
 R3 = H, alkyl, or substituted alkyl
 R4 = H, alkyl, or substituted alkyl

stereochemistry is R, S, or racemic

R2 is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic

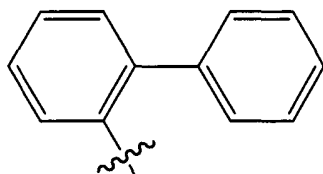
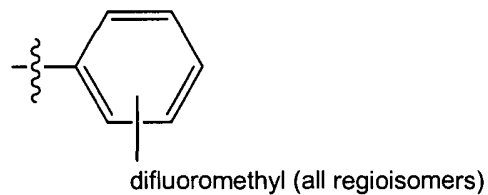
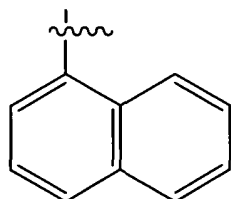
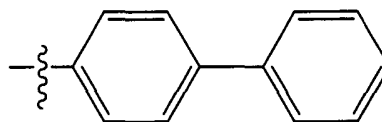
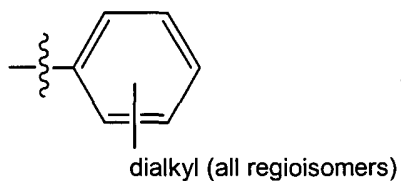
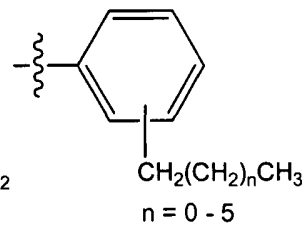
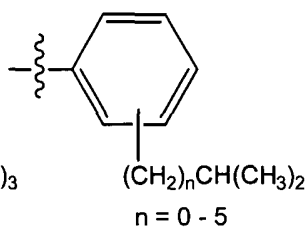
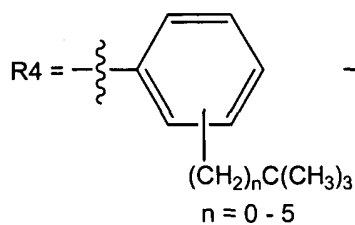


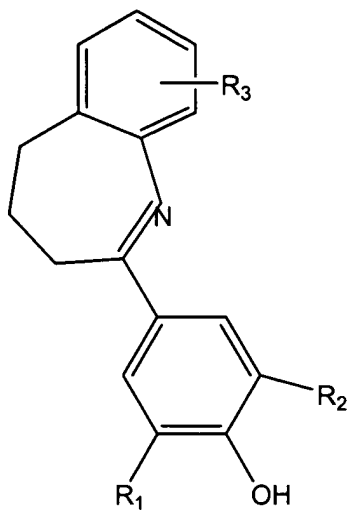


R2 = H, alkyl, or substituted alkyl

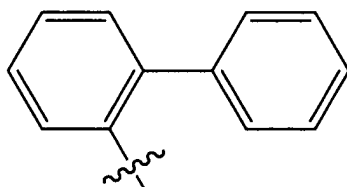
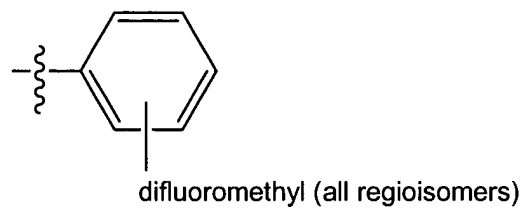
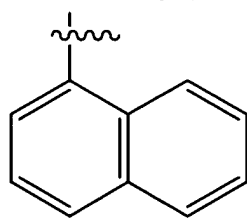
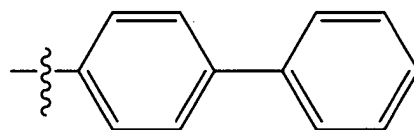
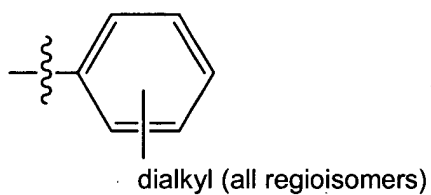
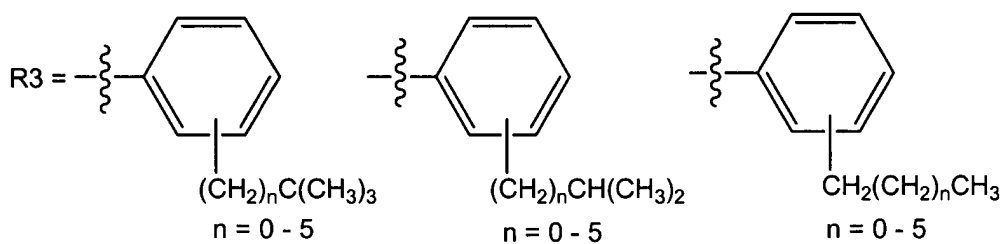
R3 = H, alkyl, or substituted alkyl

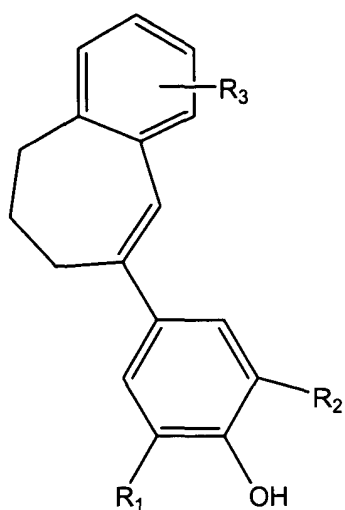
R1 is selected hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic



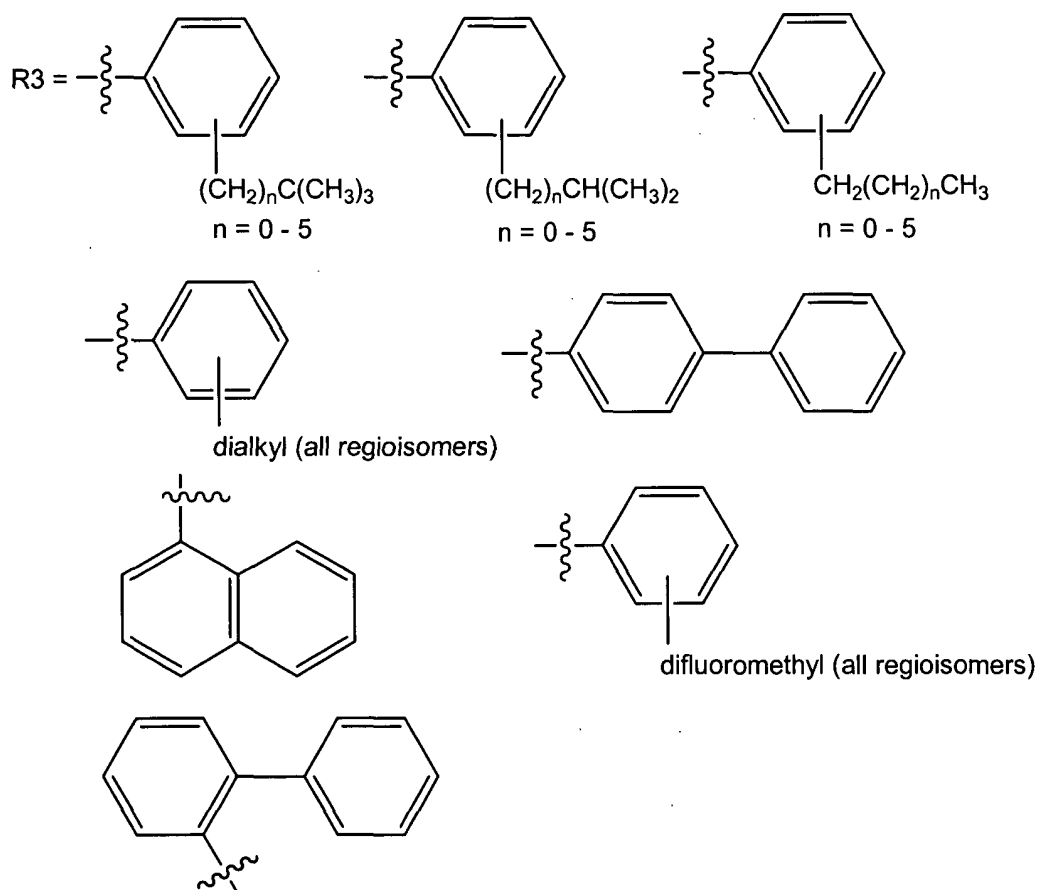


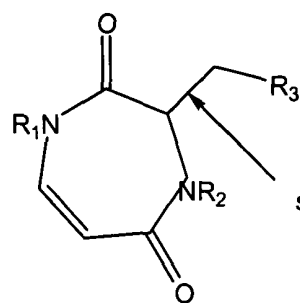
R1 = H, alkyl, or substituted alkyl
R2 = H, alkyl, or substituted alkyl





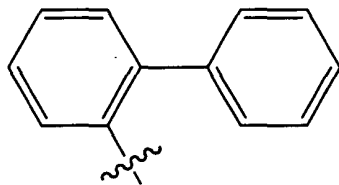
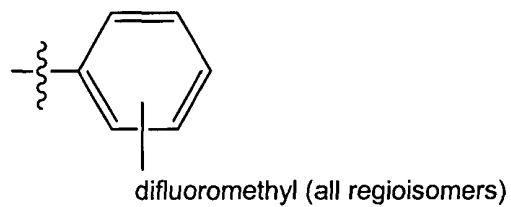
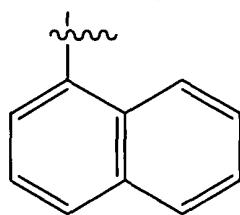
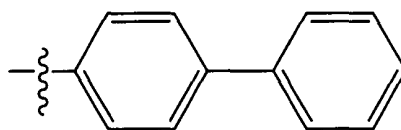
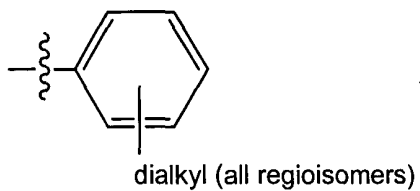
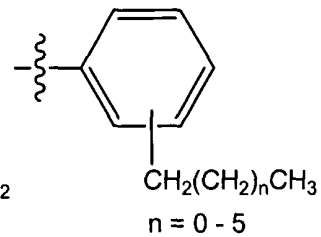
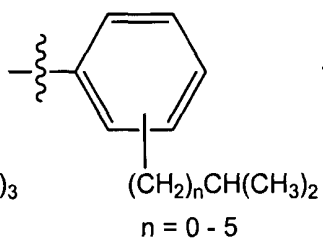
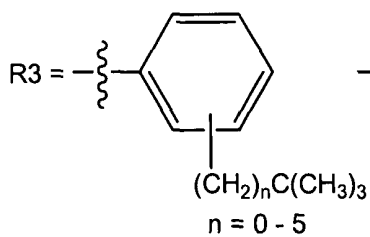
R1 = H, alkyl, or substituted alkyl
R2 = H, alkyl, or substituted alkyl

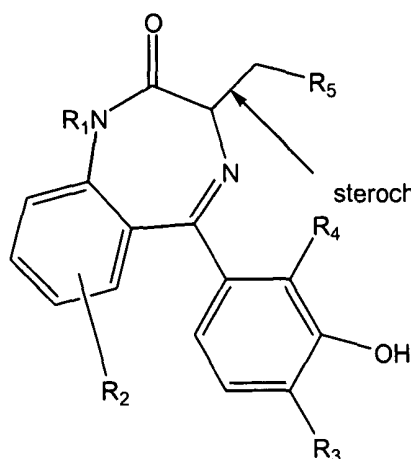




R1 = H, alkyl, or substituted alkyl
R2 = H, alkyl, or substituted alkyl

stereochemistry is R, S, or racemic





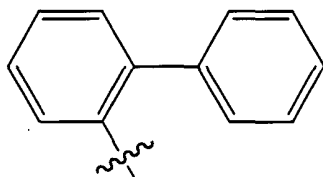
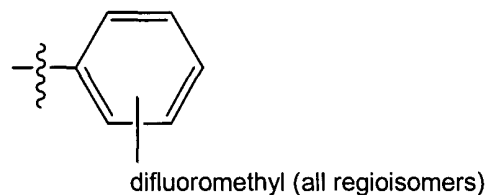
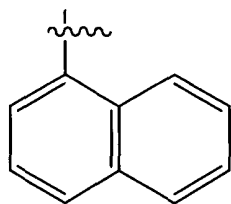
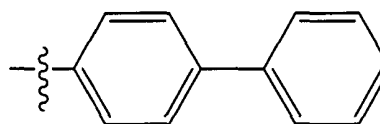
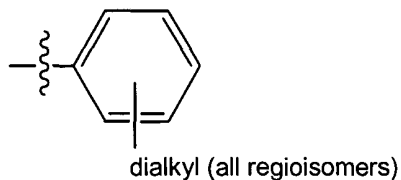
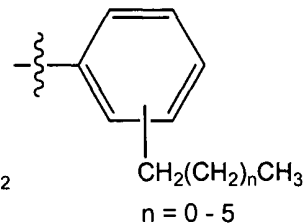
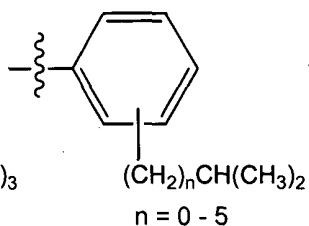
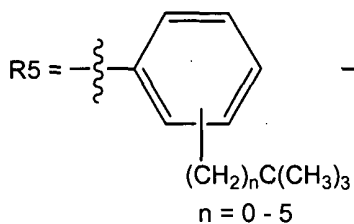
R1 = H, alkyl, or substituted alkyl

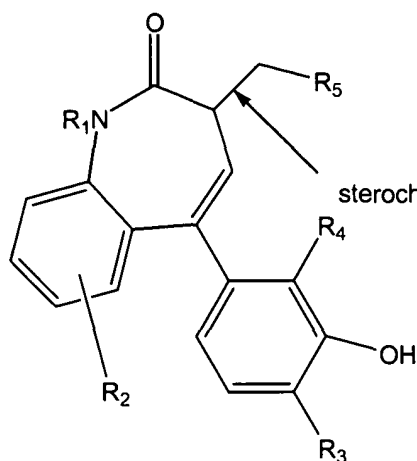
R3 = H, alkyl, or substituted alkyl

R4 = H, alkyl, or substituted alkyl

stereochemistry is R, S, or racemic

R2 is selected from hydrogen, hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic

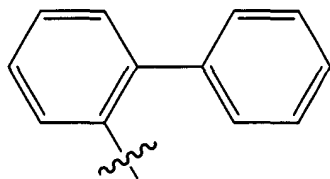
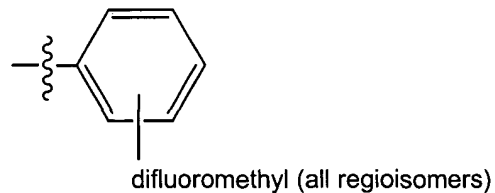
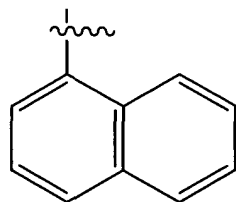
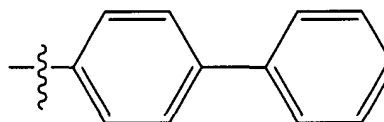
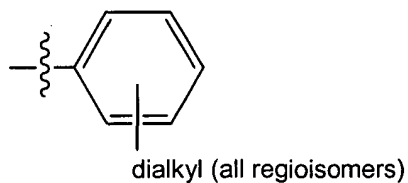
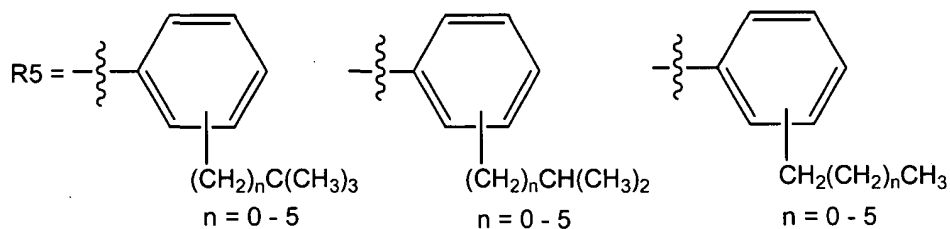


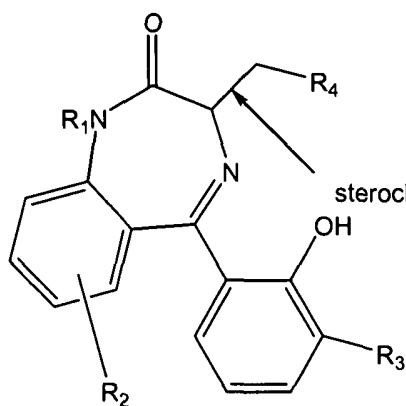


R1 = H, alkyl, or substituted alkyl
 R3 = H, alkyl, or substituted alkyl
 R4 = H, alkyl, or substituted alkyl

stereochemistry is R, S, or racemic

R2 is selected from hydrogen, hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic

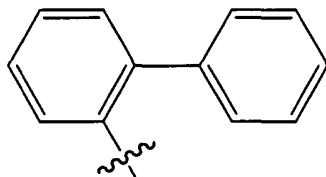
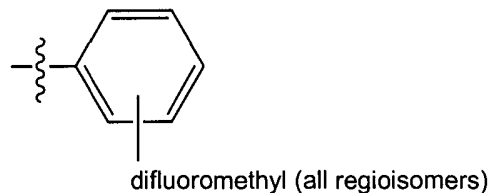
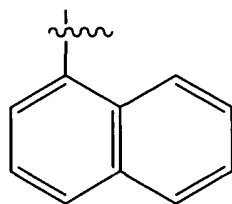
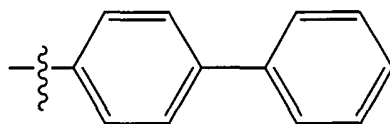
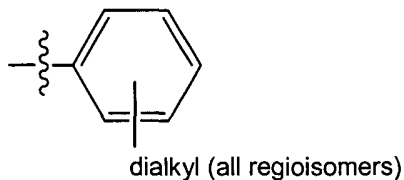
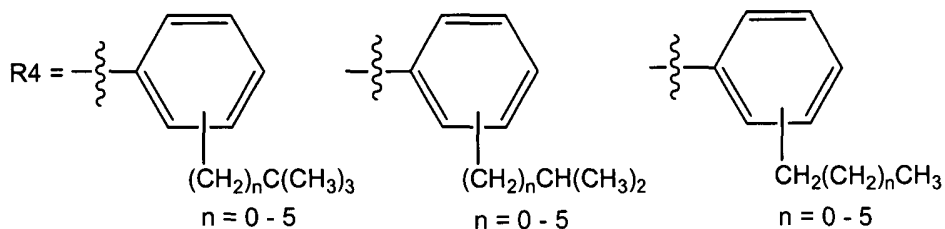


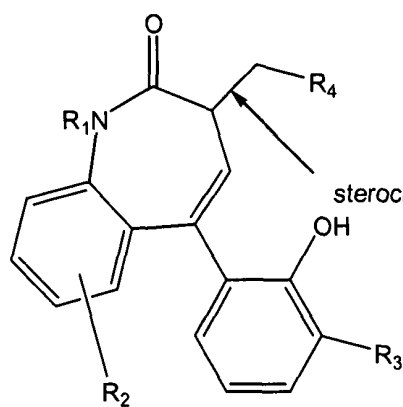


R1 = H, alkyl, or substituted alkyl
R3 = H, alkyl, or substituted alkyl

stereochemistry is R, S, or racemic

R2 is selected from hydrogen, hydroxy, an alkoxy, a halo, an amino, a lower-alkyl, a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic

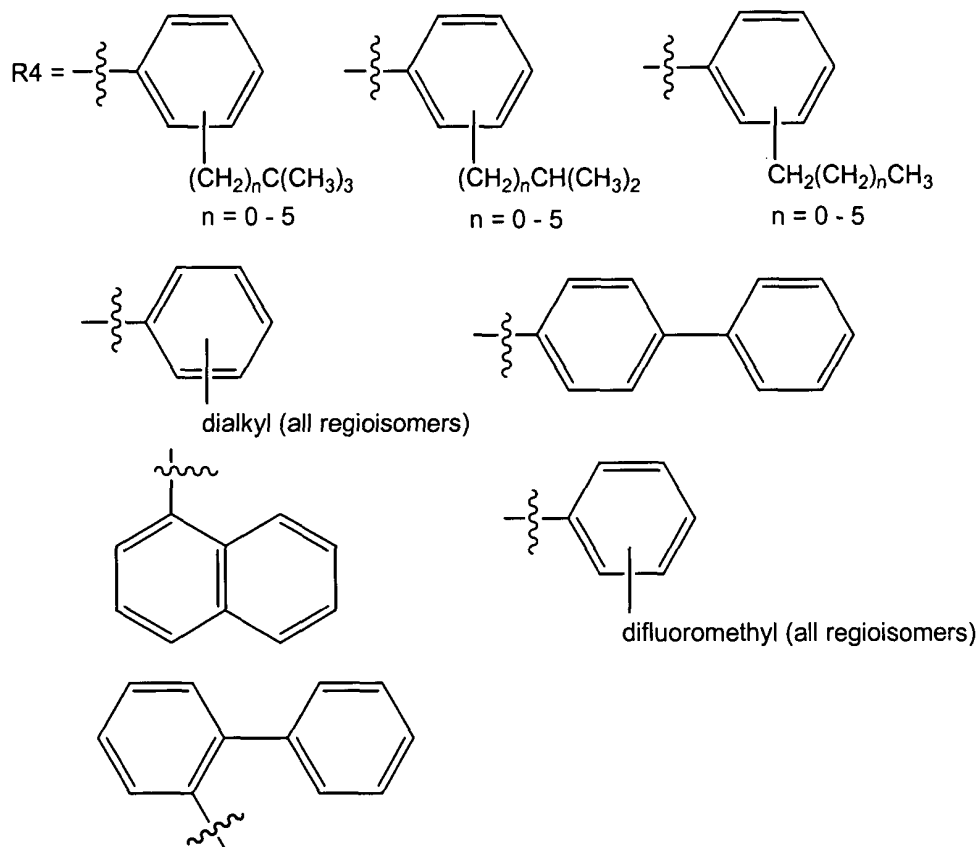


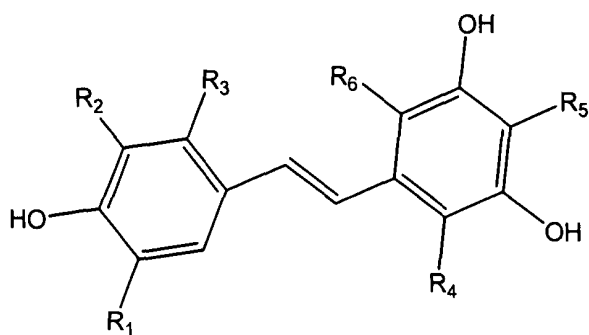


R₁ = H, alkyl, or substituted alkyl
 R₃ = H, alkyl, or substituted alkyl

stereochemistry is R, S, or racemic

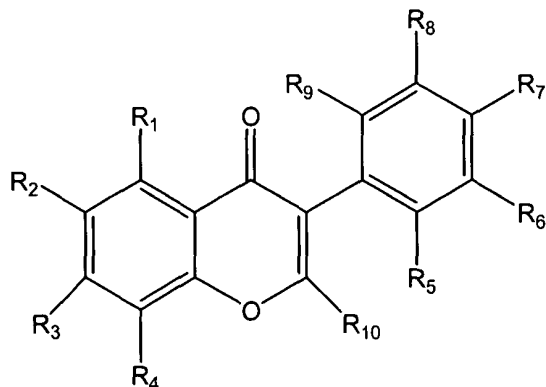
R₂ is selected from hydrogen, hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic



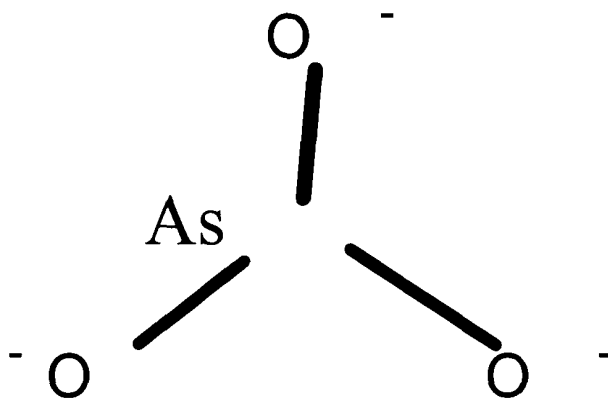


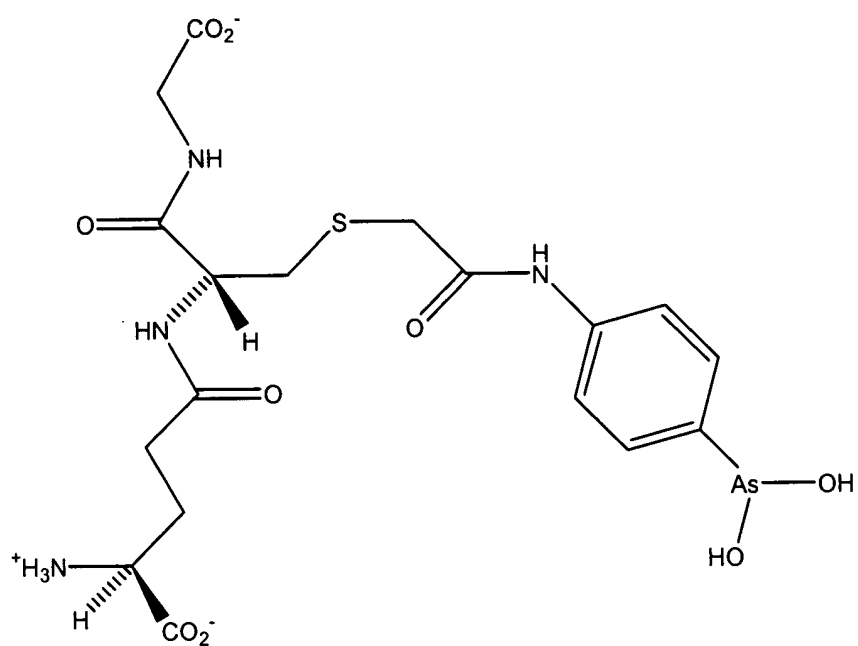
R1 is H or hydroxy

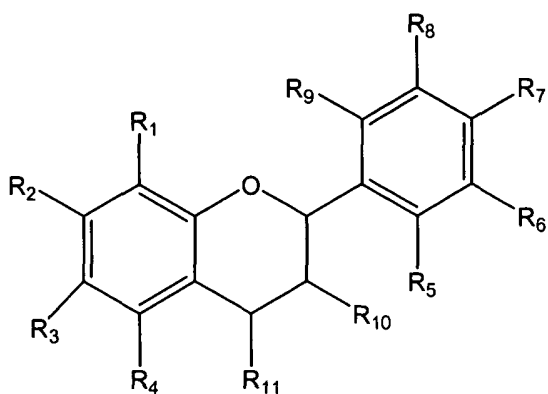
Each of R2 through R6 may be the same or different and is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic



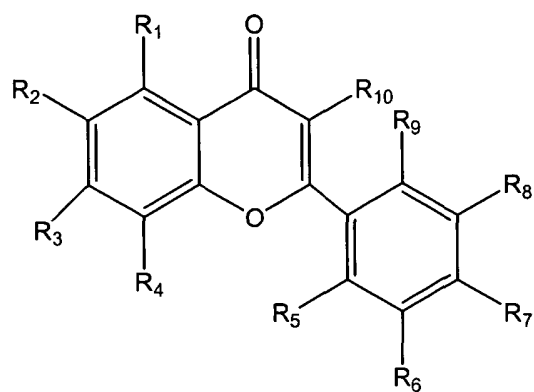
Each of R1 through R10 may be the same or different and is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic



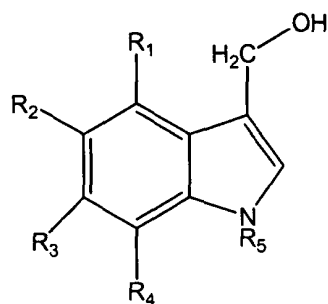




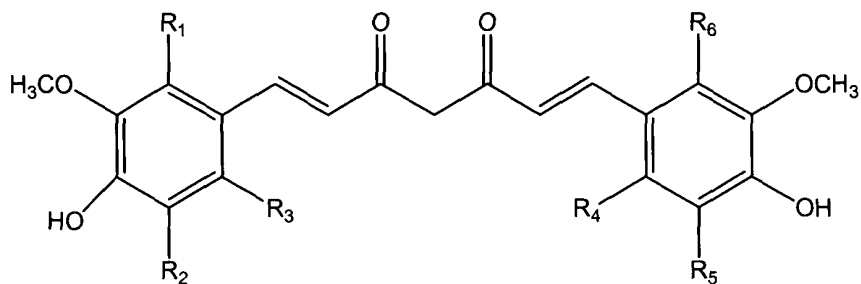
Each of R1 through R11 may be the same or different and is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic



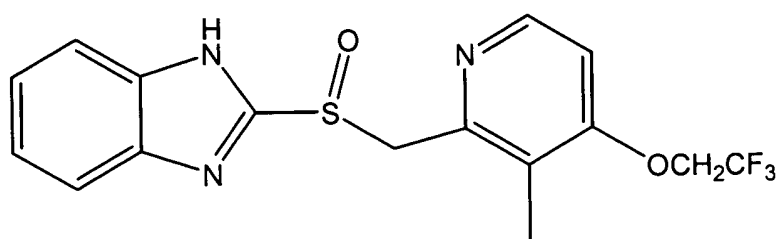
Each of R1 through R10 may be the same or different and is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic



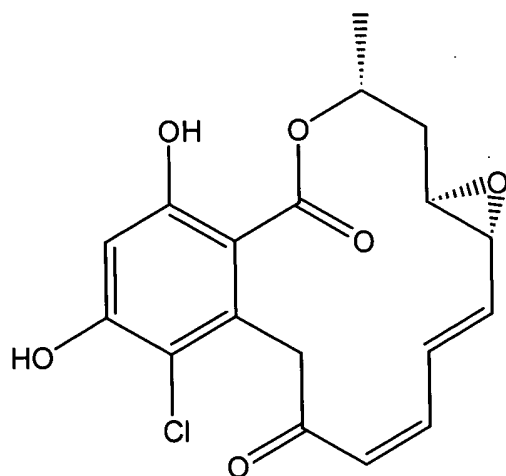
Each of R1 through R10 may be the same or different and is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic



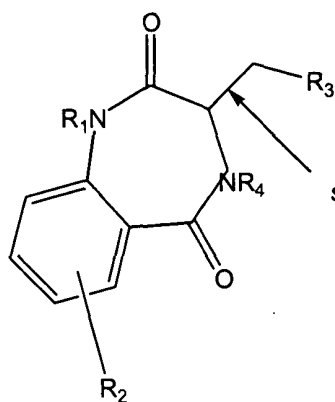
Each of R1 through R6 may be the same or different and is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic



lansoprazole



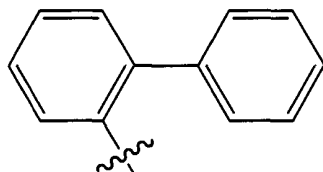
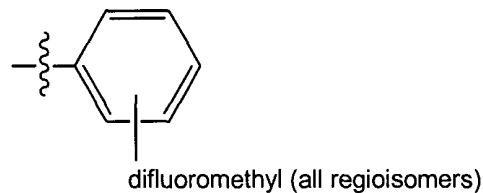
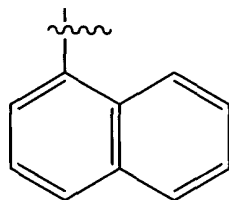
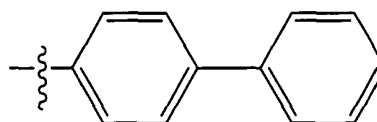
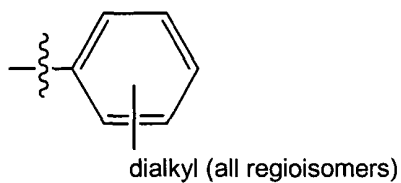
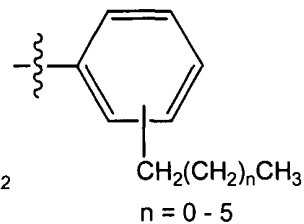
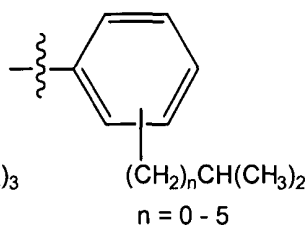
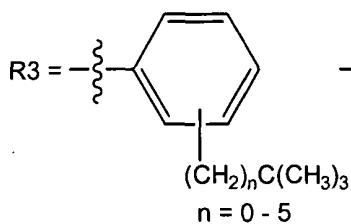
radicicol

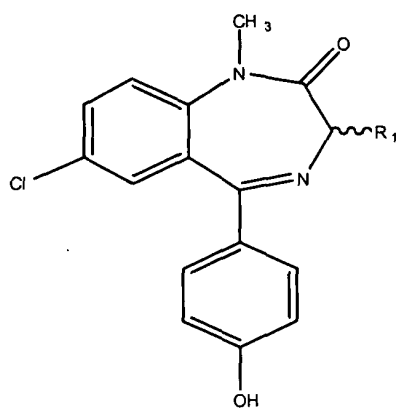


R1 = H, alkyl, or substituted alkyl
R4 = H, alkyl, or substituted alkyl

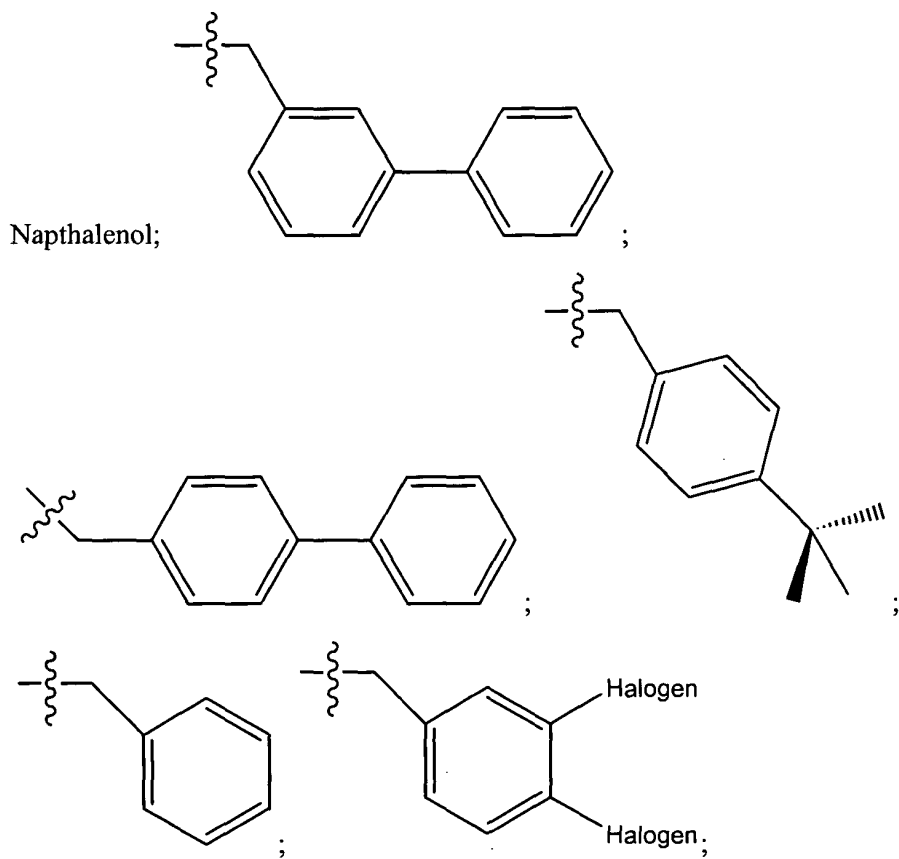
stereochemistry is R, S, or racemic

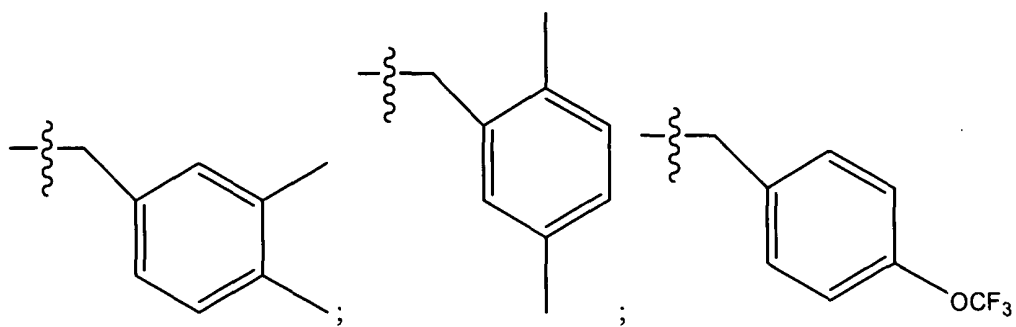
R2 is selected from hydrogen, a hydroxy, an alkoxy, a halo, an amino, a lower-alkyl-a substituted-amino, an acetylamino, a hydroxyamino, an aliphatic group having 1-8 carbons and 1-20 hydrogens, a substituted aliphatic group of similar size, a cycloaliphatic group consisting of < 10 carbons, a substituted cycloaliphatic group, an aryl, and a heterocyclic





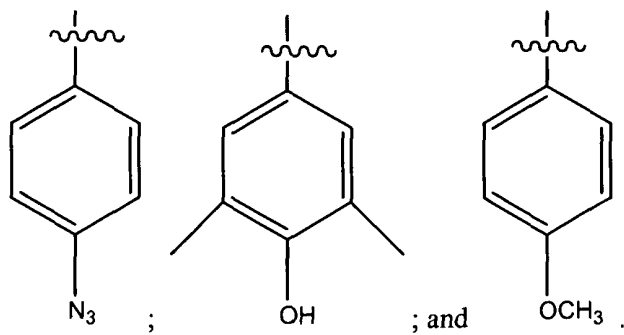
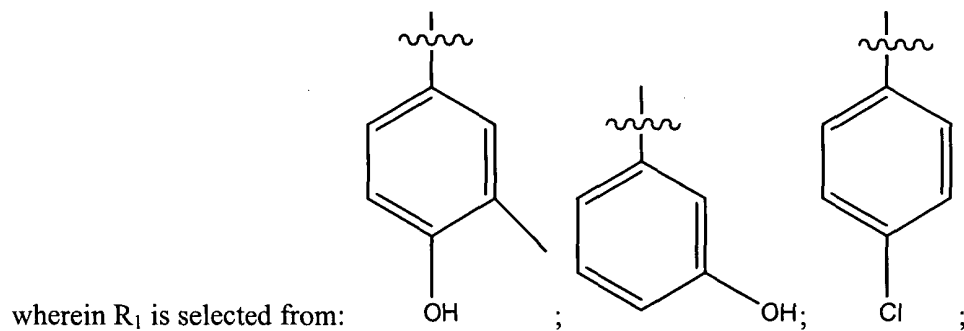
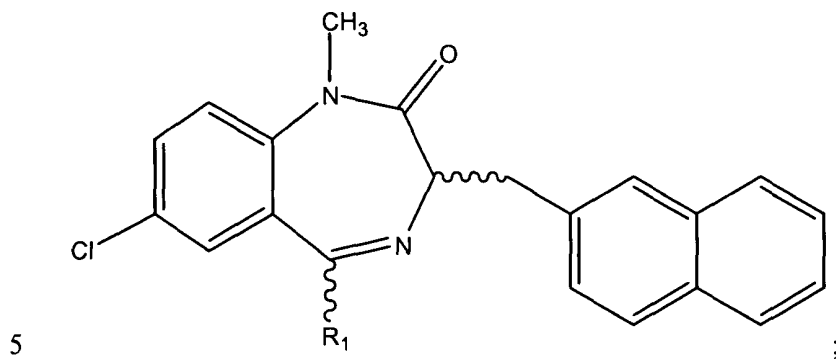
wherein R_1 is selected from naphthalaniline; phenol; 1-Napthalenol; 2-





and quinolines.

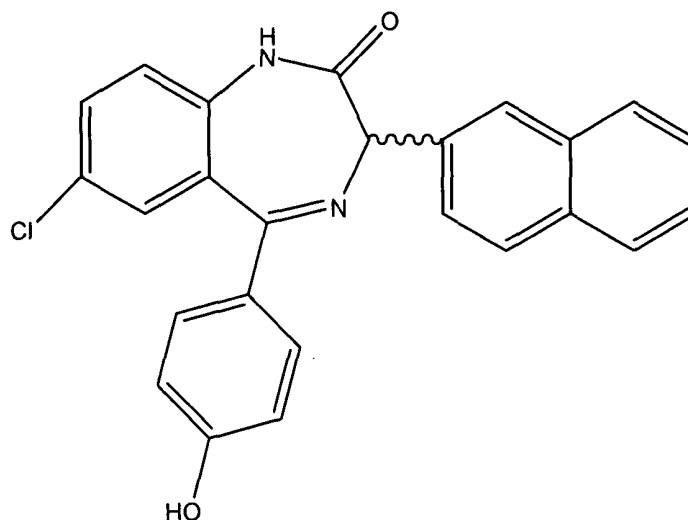
A composition comprising the following formula:



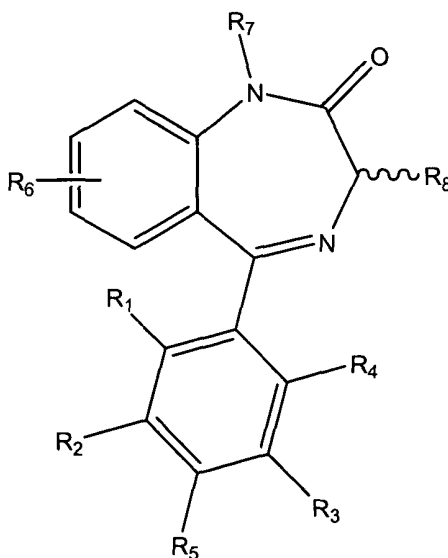
The stereochemistry of all derivatives embodied in the present invention is R, S, or racemic.

Additional specific benzodiazepine derivative examples of the present invention include the following:

5 A composition, comprising the following formula:



A composition comprising the following formula:



10 wherein R₁, R₂, R₃ and R₄ are selected from the group consisting of: hydrogen; CH₃; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one hydroxy subgroup; a linear or branched, saturated or

unsaturated aliphatic chain having at least 2 carbons, and having at least one thiol
 subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least
 2 carbons, wherein said aliphatic chain terminates with an aldehyde subgroup; a linear
 or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 5 having at least one ketone subgroup; a linear or branched, saturated or unsaturated
 aliphatic chain having at least 2 carbons; wherein said aliphatic chain terminates with
 a carboxylic acid subgroup; a linear or branched, saturated or unsaturated aliphatic
 chain having at least 2 carbons, and having at least one amide subgroup; a linear or
 branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 10 having at least one acyl group; a linear or branched, saturated or unsaturated aliphatic
 chain having at least 2 carbons, and having at least one nitrogen containing moiety
 (e.g., nitro, nitrile, etc.); a linear or branched, saturated or unsaturated aliphatic chain
 having at least 2 carbons, and having at least one amine subgroup; a linear or
 branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 15 having at least one ether subgroup; a linear or branched, saturated or unsaturated
 aliphatic chain having at least 2 carbons, and having at least one halogen subgroup; a
 linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons,
 and having at least one nitronium subgroup; wherein R5 is selected from the group
 consisting of: OH; NO₂; NR'; OR'; wherein R' is selected from the group consisting
 20 of: a linear or branched, saturated or unsaturated aliphatic chain having at least one
 carbon; a linear or branched, saturated or unsaturated aliphatic chain having at least 2
 carbons, and having at least one hydroxyl subgroup; a linear or branched, saturated or
 unsaturated aliphatic chain having at least 2 carbons, and having at least one thiol
 subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least
 25 2 carbons, wherein said aliphatic chain terminates with an aldehyde subgroup; a linear
 or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 having at least one ketone subgroup; a linear or branched, saturated or unsaturated
 aliphatic chain having at least 2 carbons; wherein said aliphatic chain terminates with
 a carboxylic acid subgroup; a linear or branched, saturated or unsaturated aliphatic
 30 chain having at least 2 carbons, and having at least one amide subgroup; a linear or
 branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and
 having at least one acyl group; a linear or branched, saturated or unsaturated aliphatic

chain having at least 2 carbons, and having at least one nitrogen containing moiety (e.g., nitro, nitrile, etc.); a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one amine subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one halogen subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one nitronium subgroup; wherein R6 is selected from the group consisting of: Hydrogen; NO₂; Cl; F; Br; I; SR'; and NR'₂; wherein R' is defined as above in R5; wherein R7 is selected from the group consisting of: Hydrogen; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons; and wherein R8 is an aliphatic cyclic group larger than benzene; wherein said larger than benzene comprises any chemical group containing 7 or more non-hydrogen atoms, and is an aryl or aliphatic cyclic group. In some embodiments, R' is any functional group that protects the oxygen of R5 from metabolism in vivo, until the compound reaches its biological target (e.g., mitochondria). In some embodiments, R' protecting group(s) is metabolized at the target site, converting R5 to a hydroxyl group.

In summary, a large number of benzodiazepine compounds and related compounds are presented herein. Any one or more of these compounds can be used to treat a variety of dysregulatory disorders related to cellular death as described elsewhere herein. The above-described compounds can also be used in drug screening assays and other diagnostic methods.

V. Pharmaceutical compositions, formulations, and exemplary administration routes and dosing considerations

Exemplary embodiments of various contemplated medicaments and pharmaceutical compositions are provided below.

A. Preparing Medicaments

The compounds of the present invention are useful in the preparation of medicaments to treat a variety of conditions associated with dysregulation of cell death, aberrant cell growth and hyperproliferation.

In addition, the compounds are also useful for preparing medicaments for treating other disorders wherein the effectiveness of the compounds are known or predicted. Such disorders include, but are not limited to, neurological (*e.g.*, epilepsy) or neuromuscular disorders. The methods and techniques for preparing medicaments of a compound are well-known in the art. Exemplary pharmaceutical formulations and routes of delivery are described below.

One of skill in the art will appreciate that any one or more of the compounds described herein, including the many specific embodiments, are prepared by applying standard pharmaceutical manufacturing procedures. Such medicaments can be delivered to the subject by using delivery methods that are well-known in the pharmaceutical arts.

B. Exemplary pharmaceutical compositions and formulation

In some embodiments of the present invention, the compositions are administered alone, while in some other embodiments, the compositions are preferably present in a pharmaceutical formulation comprising at least one active ingredient/agent (*e.g.*, benzodiazepine derivative), as defined above, together with a solid support or alternatively, together with one or more pharmaceutically acceptable carriers and optionally other therapeutic agents. Each carrier must be "acceptable" in the sense that it is compatible with the other ingredients of the formulation and not injurious to the subject.

Contemplated formulations include those suitable oral, rectal, nasal, topical (including transdermal, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary administration. In some embodiments, formulations are conveniently presented in unit dosage form and are prepared by any method known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association (*e.g.*, mixing) the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, wherein each preferably contains a predetermined amount of the active ingredient; as a powder or granules; as a solution or suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. In other embodiments, the active ingredient is presented as a bolus, electuary, or paste, *etc.*

In some embodiments, tablets comprise at least one active ingredient and optionally one or more accessory agents/carriers are made by compressing or molding the respective agents. In preferred embodiments, compressed tablets are prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (*e.g.*, povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (*e.g.*, sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets are made by molding in a suitable machine a mixture of the powdered compound (*e.g.*, active ingredient) moistened with an inert liquid diluent. Tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

Pharmaceutical compositions for topical administration according to the present invention are optionally formulated as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. In alternatively embodiments, topical formulations comprise patches or dressings such as a bandage or adhesive plasters impregnated with active ingredient(s), and optionally one or more excipients or diluents. In preferred embodiments, the topical formulations include a

compound(s) that enhances absorption or penetration of the active agent(s) through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide (DMSO) and related analogues.

If desired, the aqueous phase of a cream base includes, for example, at least
5 about 30% w/w of a polyhydric alcohol, *i.e.*, an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof.

In some embodiments, oily phase emulsions of this invention are constituted from known ingredients in a known manner. This phase typically comprises an lone
10 emulsifier (otherwise known as an emulgent), it is also desirable in some embodiments for this phase to further comprises a mixture of at least one emulsifier with a fat or an oil or with both a fat and an oil.

Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier so as to act as a stabilizer. In some embodiments it is also preferable to
15 include both an oil and a fat. Together, the emulsifier(s) with or without stabilizer(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

Emulgents and emulsion stabilizers suitable for use in the formulation of the
20 present invention include Tween 60, Span 80, cetostearyl alcohol, myristyl alcohol, glyceryl monostearate and sodium lauryl sulfate.

The choice of suitable oils or fats for the formulation is based on achieving the desired properties (*e.g.*, cosmetic properties), since the solubility of the active compound/agent in most oils likely to be used in pharmaceutical emulsion
25 formulations is very low. Thus creams should preferably be a non-greasy, non-staining and washable products with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl
30 palmitate or a blend of branched chain esters known as Crodamol CAP may be used, the last three being preferred esters. These may be used alone or in combination

depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

Formulations suitable for topical administration to the eye also include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier,
5 especially an aqueous solvent for the agent.

Formulations for rectal administration may be presented as a suppository with suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as pessaries, creams, gels, pastes, foams or spray formulations containing in addition to
10 the agent, such carriers as are known in the art to be appropriate.

Formulations suitable for nasal administration, wherein the carrier is a solid, include coarse powders having a particle size, for example, in the range of about 20 to about 500 microns which are administered in the manner in which snuff is taken, *i.e.*, by rapid inhalation (*e.g.*, forced) through the nasal passage from a container of the
15 powder held close up to the nose. Other suitable formulations wherein the carrier is a liquid for administration include, but are not limited to, nasal sprays, drops, or aerosols by nebulizer, and include aqueous or oily solutions of the agents.

Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain antioxidants, buffers,
20 bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. In some embodiments, the formulations are
25 presented/formulated in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind
30 previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily subdose, as herein above-recited, or an appropriate fraction thereof, of an agent.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include such further agents as
5 sweeteners, thickeners and flavoring agents. It also is intended that the agents, compositions and methods of this invention be combined with other suitable compositions and therapies. Still other formulations optionally include food additives (suitable sweeteners, flavorings, colorings, *etc.*), phytonutrients (*e.g.*, flax seed oil), minerals (*e.g.*, Ca, Fe, K, *etc.*), vitamins, and other acceptable compositions (*e.g.*,
10 conjugated linoelic acid), extenders, and stabilizers, *etc.*

C. Exemplary administration routes and dosing considerations

Various delivery systems are known and can be used to administer a therapeutic agents (*e.g.*, benzodiazepine derivatives) of the present invention, *e.g.*,
15 encapsulation in liposomes, microparticles, microcapsules, receptor-mediated endocytosis, and the like. Methods of delivery include, but are not limited to, intra-arterial, intra-muscular, intravenous, intranasal, and oral routes. In specific embodiments, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for
20 example, and not by way of limitation, local infusion during surgery, injection, or by means of a catheter.

The agents identified herein as effective for their intended purpose can be administered to subjects or individuals susceptible to or at risk of developing pathological growth of target cells and condition correlated with this. When the agent
25 is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject. To determine patients that can be beneficially treated, a tissue sample is removed from the patient and the cells are assayed for sensitivity to the agent.

30 Therapeutic amounts are empirically determined and vary with the pathology being treated, the subject being treated and the efficacy and toxicity of the agent. When delivered to an animal, the method is useful to further confirm efficacy of the

agent. One example of an animal model is MLR/MpJ-*lpr/lpr* ("MLR-*lpr*") (available from Jackson Laboratories, Bal Harbor, Maine). MLR-*lpr* mice develop systemic autoimmune disease. Alternatively, other animal models can be developed by inducing tumor growth, for example, by subcutaneously inoculating nude mice with about 10^5 to about 10^9 hyperproliferative, cancer or target cells as defined herein. When the tumor is established, the compounds described herein are administered, for example, by subcutaneous injection around the tumor. Tumor measurements to determine reduction of tumor size are made in two dimensions using venier calipers twice a week. Other animal models may also be employed as appropriate. Such animal models for the above-described diseases and conditions are well-known in the art.

In some embodiments, *in vivo* administration is effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations are carried out with the dose level and pattern being selected by the treating physician.

Suitable dosage formulations and methods of administering the agents are readily determined by those of skill in the art. Preferably, the compounds are administered at about 0.01 mg/kg to about 200 mg/kg, more preferably at about 0.1 mg/kg to about 100 mg/kg, even more preferably at about 0.5 mg/kg to about 50 mg/kg. When the compounds described herein are co-administered with another agent (*e.g.*, as sensitizing agents), the effective amount may be less than when the agent is used alone.

The pharmaceutical compositions can be administered orally, intranasally, parenterally or by inhalation therapy, and may take the form of tablets, lozenges, granules, capsules, pills, ampoules, suppositories or aerosol form. They may also take the form of suspensions, solutions and emulsions of the active ingredient in aqueous or nonaqueous diluents, syrups, granulates or powders. In addition to an agent of the present invention, the pharmaceutical compositions can also contain other pharmaceutically active compounds or a plurality of compounds of the invention.

More particularly, an agent of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including, but not limited to, oral, rectal, nasal, topical (including, but not limited to, transdermal, aerosol, buccal and sublingual), vaginal, parental (including, but not limited to, subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It is also appreciated that the preferred route varies with the condition and age of the recipient, and the disease being treated.

Ideally, the agent should be administered to achieve peak concentrations of the active compound at sites of disease. This may be achieved, for example, by the intravenous injection of the agent, optionally in saline, or orally administered, for example, as a tablet, capsule or syrup containing the active ingredient.

Desirable blood levels of the agent may be maintained by a continuous infusion to provide a therapeutic amount of the active ingredient within disease tissue. The use of operative combinations is contemplated to provide therapeutic combinations requiring a lower total dosage of each component antiviral agent than may be required when each individual therapeutic compound or drug is used alone, thereby reducing adverse effects.

D. Exemplary co-administration routes and dosing considerations

The present invention also includes methods involving co-administration of the compounds described herein with one or more additional active agents. Indeed, it is a further aspect of this invention to provide methods for enhancing prior art therapies and/or pharmaceutical compositions by co-administering a compound of this invention. In co-administration procedures, the agents may be administered concurrently or sequentially. In one embodiment, the compounds described herein are administered prior to the other active agent(s). The pharmaceutical formulations and modes of administration may be any of those described above. In addition, the two or more co-administered chemical agents, biological agents or radiation may each be administered using different modes or different formulations.

The agent or agents to be co-administered depends on the type of condition being treated. For example, when the condition being treated is cancer, the additional agent can be a chemotherapeutic agent or radiation. When the condition being treated

is an autoimmune disorder, the additional agent can be an immunosuppressant or an anti-inflammatory agent. When the condition being treated is chronic inflammation, the additional agent can be an anti-inflammatory agent. The additional agents to be co-administered, such as anticancer, immunosuppressant, anti-inflammatory, and can
5 be any of the well-known agents in the art, including, but not limited to, those that are currently in clinical use. The determination of appropriate type and dosage of radiation treatment is also within the skill in the art or can be determined with relative ease.

Treatment of the various conditions associated with abnormal apoptosis is
10 generally limited by the following two major factors: (1) the development of drug resistance and (2) the toxicity of known therapeutic agents. In certain cancers, for example, resistance to chemicals and radiation therapy has been shown to be associated with inhibition of apoptosis. Some therapeutic agents have deleterious side effects, including non-specific lymphotoxicity, renal and bone marrow toxicity.

15 The methods described herein address both these problems. Drug resistance, where increasing dosages are required to achieve therapeutic benefit, is overcome by co-administering the compounds described herein with the known agent. The compounds described herein appear to sensitize target cells to known agents (and vice versa) and, accordingly, less of these agents are needed to achieve a therapeutic
20 benefit.

The sensitizing function of the claimed compounds also addresses the problems associated with toxic effects of known therapeutics. In instances where the known agent is toxic, it is desirable to limit the dosages administered in all cases, and particularly in those cases where drug resistance has increased the requisite dosage.
25 When the claimed compounds are co-administered with the known agent, they reduce the dosage required which, in turn, reduces the deleterious effects. Further, because the claimed compounds are themselves both effective and non-toxic in large doses, co-administration of proportionally more of these compounds than known toxic therapeutics will achieve the desired effects while minimizing toxic effects.

30

VI. Drug screens

In preferred embodiments of the present invention, the compounds of the present invention, and other potentially useful compounds, are screened for their binding affinity to the oligomycin sensitivity conferring protein (OSCP) portion of the mitochondrial ATP synthase complex. In particularly preferred embodiments, compounds are selected for use in the methods of the present invention by measuring their binding affinity to recombinant OSCP protein. A number of suitable screens for measuring the binding affinity of drugs and other small molecules to receptors are known in the art. In some embodiments, binding affinity screens are conducted in *in vitro* systems. In other embodiments, these screens are conducted in *in vivo* or *ex vivo* systems. While in some embodiments quantifying the intracellular level of ATP following administration of the compounds of the present invention provides an indication of the efficacy of the methods, preferred embodiments of the present invention do not require intracellular ATP or pH level quantification.

Additional embodiments are directed to measuring levels (*e.g.*, intracellular) of superoxide in cells and/or tissues to measure the effectiveness of particular contemplated methods and compounds of the present invention. In this regard, those skilled in the art will appreciate and be able to provide a number of assays and methods useful for measuring superoxide levels in cells and/or tissues.

In some embodiments, structure-based virtual screening methodologies are contemplated for predicting the binding affinity of compounds of the present invention with OSCP.

Any suitable assay that allows for a measurement of the rate of binding or the affinity of a benzodiazepine or other compound to the OSCP may be utilized. Examples include, but are not limited to, competition binding using Bz-423, surface plasma resonance (SPR) and radio-immunoprecipitation assays (Lowman *et al.*, J. Biol.Chem. 266:10982 [1991]). Surface Plasmon Resonance techniques involve a surface coated with a thin film of a conductive metal, such as gold, silver, chrome or aluminum, in which electromagnetic waves, called Surface Plasmons, can be induced by a beam of light incident on the metal glass interface at a specific angle called the Surface Plasmon Resonance angle. Modulation of the refractive index of the interfacial region between the solution and the metal surface following binding of the

captured macromolecules causes a change in the SPR angle which can either be measured directly or which causes the amount of light reflected from the underside of the metal surface to change. Such changes can be directly related to the mass and other optical properties of the molecules binding to the SPR device surface. Several biosensor systems based on such principles have been disclosed (See e.g., WO 90/05305). There are also several commercially available SPR biosensors (e.g., BiaCore, Uppsala, Sweden).

In some embodiments, compounds are screened in cell culture or *in vivo* (e.g., non-human or human mammals) for their ability to modulate mitochondrial ATP synthase activity. Any suitable assay may be utilized, including, but not limited to, cell proliferation assays (Commercially available from, e.g., Promega, Madison, WI and Stratagene, La Jolla, CA) and cell based dimerization assays. (See e.g., Fuh *et al.*, Science, 256:1677 [1992]; Colosi *et al.*, J. Biol. Chem., 268:12617 [1993]). Additional assay formats that find use with the present invention include, but are not limited to, assays for measuring cellular ATP levels, and cellular superoxide levels.

The present invention also provides methods of modifying and derivatizing the compositions of the present invention to increase desirable properties (e.g., binding affinity, activity, and the like), or to minimize undesirable properties (e.g., nonspecific reactivity, toxicity, and the like). The principles of chemical derivatization are well understood. In some embodiments, iterative design and chemical synthesis approaches are used to produce a library of derivatized child compounds from a parent compound. In other embodiments, rational design methods are used to predict and model *in silico* ligand-receptor interactions prior to confirming results by routine experimentation.

VII. Therapeutic Application

A. General Therapeutic Application

In particularly preferred embodiments, the compositions (e.g., benzodiazepine derivatives) of the present invention provide therapeutic benefits to patients suffering from any one or more of a number of conditions (e.g., diseases characterized by dysregulation of necrosis and/or apoptosis processes in a cell or tissue, disease characterized by aberrant cell growth and/or hyperproliferation, *etc.*) by modulating

(*e.g.*, inhibiting or promoting) the activity of the mitochondrial ATP synthase (as referred to as mitochondrial F_0F_1 ATPase) complexes in affected cells or tissues. In further preferred embodiments, the compositions of the present invention are used to treat autoimmune/chronic inflammatory conditions (*e.g.*, psoriasis). In even further
5 embodiments, the compositions of the present invention are used in conjunction with stenosis therapy to treat compromised (*e.g.*, occluded) vessels.

 In particularly preferred embodiments, the compositions of the present invention inhibit the activity of mitochondrial ATP synthase complex by binding to a specific subunit of this multi-subunit protein complex. While the present invention is
10 not limited to any particular mechanism, nor to any understanding of the action of the agents being administered, in some embodiments, the compositions of the present invention bind to the oligomycin sensitivity conferring protein (OSCP) portion of the mitochondrial ATP synthase complex. Likewise, it is further contemplated that when the compositions of the present invention bind to the OSCP the initial affect is overall
15 inhibition of the mitochondrial ATP synthase complex, and that the downstream consequence of binding is a change in ATP or pH level and the production of reactive oxygen species (*e.g.*, O_2^-). In still other preferred embodiments, while the present invention is not limited to any particular mechanism, nor to any understanding of the action of the agents being administered, it is contemplated that the generation of free
20 radicals ultimately results in cell killing. In yet other embodiments, while the present invention is not limited to any particular mechanism, nor to any understanding of the action of the agents being administered, it is contemplated that the inhibiting mitochondrial ATP synthase complex using the compositions and methods of the present invention provides therapeutically useful inhibition of cell proliferation.

25 Accordingly, preferred methods embodied in the present invention, provide therapeutic benefits to patients by providing compounds of the present invention that modulate (*e.g.*, inhibiting or promoting) the activity of the mitochondrial ATP synthase complexes in affected cells or tissues via binding to the oligomycin sensitivity conferring protein (OSCP) portion of the mitochondrial ATP synthase
30 complex. Importantly, by itself the OSCP has no biological activity.

 Thus, in one broad sense, preferred embodiments of the present invention are directed to the discovery that many diseases characterized by dysregulation of

necrosis and/or apoptosis processes in a cell or tissue, or diseases characterized by aberrant cell growth and/or hyperproliferation, *etc.*, can be treated by modulating the activity of the mitochondrial ATP synthase complex including, but not limited to, by binding to the oligomycin sensitivity conferring protein (OSCP) component thereof.

5 The present invention is not intended to be limited, however, to the practice of the compositions and methods explicitly described herein. Indeed, those skilled in the art will appreciate that a number of additional compounds not specifically recited herein (*e.g.*, non-benzodiazepine derivatives) are suitable for use in the methods disclosed herein of modulating the activity of mitochondrial ATP synthase.

10 The present invention thus specifically contemplates that any number of suitable compounds presently known in the art, or developed later, can optionally find use in the methods of the present invention. For example, compounds including, but not limited to, oligomycin, ossamycin, cytovaricin, apoptolidin, bafilomycin, resveratrol, piceatannol, and dicyclohexylcarbodiimide (DCCD), and the like, find use
15 in the methods of the present invention. The present invention is not intended, however, to be limited to the methods or compounds specified above. In one embodiment, that compounds potentially useful in the methods of the present invention may be selected from those suitable as described in the scientific literature. (*See e.g.*, K.B. Wallace and A.A. Starkov, *Annu. Rev. Pharmacol. Toxicol.*, 40:353-
20 388 [2000]; A.R. Solomon *et al.*, *Proc. Nat. Acad. Sci. U.S.A.*, 97(26):14766-14771 [2000]).

In some embodiments, compounds potentially useful in methods of the present invention are screened against the National Cancer Institute's (NCI-60) cancer cell lines for efficacy. (*See e.g.*, A. Monks *et al.*, *J. Natl. Cancer Inst.*, 83:757-766 [1991];
25 and K.D. Paull *et al.*, *J. Natl. Cancer Inst.*, 81:1088-1092 [1989]). Additional screens suitable screens (*e.g.*, autoimmunity disease models, *etc.*) are within the skill in the art.

In one aspect, derivatives (*e.g.*, pharmaceutically acceptable salts, analogs, stereoisomers, and the like) of the exemplary compounds or other suitable compounds
30 are also contemplated as being useful in the methods of the present invention.

In other preferred embodiments, the compositions of the present invention are used in conjunction with stenosis therapy to treat compromised (*e.g.*, occluded)

vessels. In further embodiments, the compositions of the present invention are used in conjunction with stenosis therapy to treat compromised cardiac vessels.

Vessel stenosis is a condition that develops when a vessel (*e.g.*, aortic valve) becomes narrowed. For example, aortic valve stenosis is a heart condition that
5 develops when the valve between the lower left chamber (left ventricle) of the heart and the major blood vessel called the aorta becomes narrowed. This narrowing (*e.g.*, stenosis) creates too small a space for the blood to flow to the body. Normally the left ventricle pumps oxygen-rich blood to the body through the aorta, which branches into a system of arteries throughout the body. When the heart pumps, the 3 flaps, or
10 leaflets, of the aortic valve open one way to allow blood to flow from the ventricle into the aorta. Between heartbeats, the flaps close to form a tight seal so that blood does not leak backward through the valve. If the aortic valve is damaged, it may become narrowed (stenosed) and blood flow may be reduced to organs in the body, including the heart itself. The long-term outlook for people with aortic valve stenosis
15 is poor once symptoms develop. People with untreated aortic valve stenosis who develop symptoms of heart failure usually have a life expectancy of 3 years or less.

Several types of treatment exist for treating compromised valves (*e.g.*, balloon dilation, ablation, atherectomy or laser treatment). One type of treatment for compromised cardiac valves is angioplasty. Angioplasty involves inserting a balloon-
20 tipped tube, or catheter, into a narrow or blocked artery in an attempt to open it. By inflating and deflating the balloon several times, physicians usually are able to widen the artery.

A common limitation of angioplasty or valve expansion procedures is restenosis. Restenosis is the reclosure of a peripheral or coronary artery following
25 trauma to that artery caused by efforts to open a stenosed portion of the artery, such as, for example, by balloon dilation, ablation, atherectomy or laser treatment of the artery. For these angioplasty procedures, restenosis occurs at a rate of about 20-50% depending on the definition, vessel location, lesion length and a number of other morphological and clinical variables. Restenosis is believed to be a natural healing
30 reaction to the injury of the arterial wall that is caused by angioplasty procedures. The healing reaction begins with the thrombotic mechanism at the site of the injury. The final result of the complex steps of the healing process can be intimal hyperplasia, the

uncontrolled migration and proliferation of medial smooth muscle cells, combined with their extracellular matrix production, until the artery is again stenosed or occluded.

5 In an attempt to prevent restenosis, metallic intravascular stents have been permanently implanted in coronary or peripheral vessels. The stent is typically inserted by catheter into a vascular lumen and expanded into contact with the diseased portion of the arterial wall, thereby providing mechanical support for the lumen. However, it has been found that restenosis can still occur with such stents in place. Also, the stent itself can cause undesirable local thrombosis. To address the
10 problem of thrombosis, persons receiving stents also receive extensive systemic treatment with anticoagulant and antiplatelet drugs.

To address the restenosis problem, it has been proposed to provide stents which are seeded with endothelial cells (Dichek, D. A. et al Seeding of Intravascular Stents With Genetically Engineered Endothelial Cells; Circulation 1989; 80: 1347-
15 1353). In that experiment, sheep endothelial cells that had undergone retrovirus-mediated gene transfer for either bacterial beta-galactosidase or human tissue-type plasminogen activator were seeded onto stainless steel stents and grown until the stents were covered. The cells were therefore able to be delivered to the vascular wall where they could provide therapeutic proteins. Other methods of providing
20 therapeutic substances to the vascular wall by means of stents have also been proposed such as in international patent application WO 91/12779 "Intraluminal Drug Eluting Prosthesis" and international patent application WO 90/13332 "Stent With Sustained Drug Delivery". In those applications, it is suggested that antiplatelet agents, anticoagulant agents, antimicrobial agents, anti-inflammatory agents,
25 antimetabolic agents and other drugs could be supplied in stents to reduce the incidence of restenosis. Further, other vasoreactive agents such as nitric oxide releasing agents could also be used.

An additional cause of restenosis is the over-proliferation of treated tissue. In preferred embodiments, the anti-proliferative properties of the present invention
30 inhibit restenosis. Drug-eluting stents are well known in the art (*see, e.g.*, U.S. Patent No.: 5,697,967; U.S. Patent No.: 5,599,352; and U.S. Patent No.: 5,591,227; each of which are herein incorporated by reference). In preferred embodiments, the

compositions of the present invention are eluted from drug-eluting stents in the treatment of compromised (*e.g.*, occluded) vessels. In further embodiments, the compositions of the present invention are eluted from drug-eluting stents in the treatment of compromised cardiac vessels.

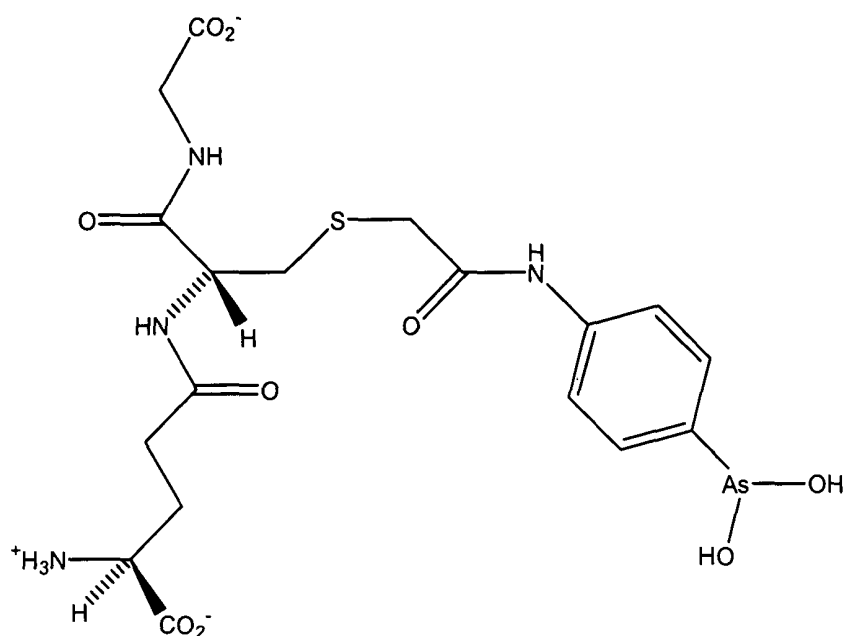
5 Those skilled in the art of preparing pharmaceutical compounds and formulations will appreciate that when selecting optional compounds for use in the methods disclosed herein, that suitability considerations include, but are not limited to, the toxicity, safety, efficacy, availability, and cost of the particular compounds.

10 **B. Autoimmune Disorder and Chronic Inflammatory Disorder
Therapeutic Application**

Autoimmune disorders and chronic inflammatory disorders often result from dysfunctional cellular proliferation regulation and/or cellular apoptosis regulation. Mitochondria perform a key role in the control and execution of cellular apoptosis.

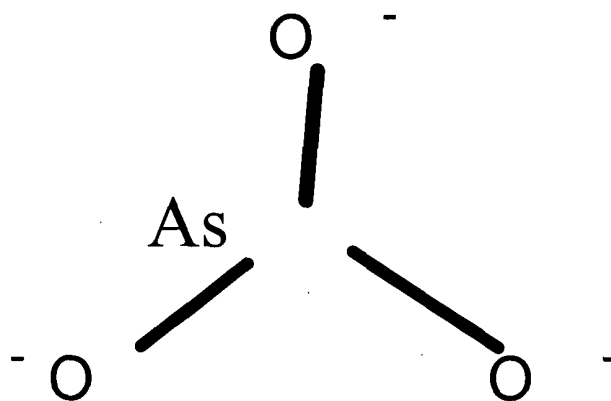
15 The mitochondrial permeability transition pore (MPTP) is a pore that spans the inner and outer mitochondrial membranes and functions in the regulation of proapoptotic particles. Transient MPTP opening results in the release of cytochrome c and the apoptosis inducing factor from the mitochondrial intermembrane space, resulting in cellular apoptosis.

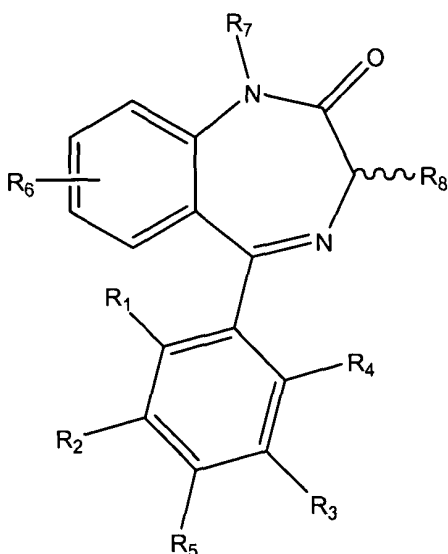
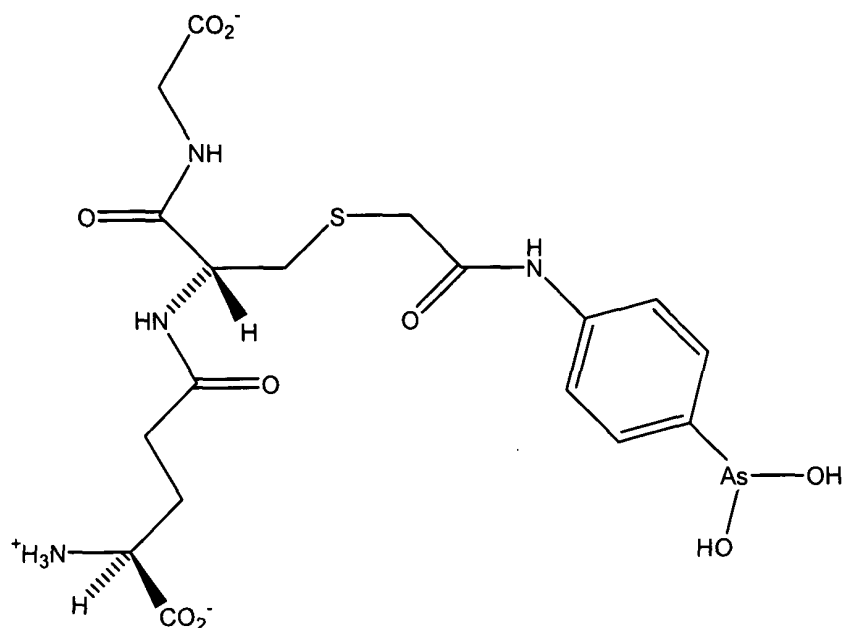
20 The oligomycin sensitivity conferring protein (OSCP) is a subunit of the F_0F_1 mitochondrial ATP synthase/ATPase and functions in the coupling of a proton gradient across the F_0 sector of the enzyme in the mitochondrial membrane. In preferred embodiments, compounds of the present invention binds the OSCP, increases superoxide and cytochrome c levels, increases cellular apoptosis, and
25 inhibits cellular proliferation. The adenine nucleotide translocator (ANT) is a 30kDa protein that spans the inner mitochondrial membrane and is central to the mitochondrial permeability transition pore (MPTP). Thiol oxidizing or alkylating agents are powerful activators of the MPTP that act by modifying one or more of three unpaired cysteines in the matrix side of the ANT. 4-(N-(S-
30 glutathionylacetyl)amino) phenylarsenoxide,



inhibits the ANT.

The compounds and methods of the present invention are useful in the treatment of autoimmune disorders and chronic inflammatory disorders. In such
 5 embodiments, the present invention provides a subject suffering from an autoimmune disorder and/or a chronic inflammatory disorder, and a composition comprising the following formula(s):





- wherein R_1 , R_2 , R_3 and R_4 are selected from the group consisting of: hydrogen;
- 5 CH_3 ; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one hydroxy subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one thiol subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least
- 10 2 carbons, wherein said aliphatic chain terminates with an aldehyde subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and

having at least one ketone subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons; wherein said aliphatic chain terminates with a carboxylic acid subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one amide subgroup; a linear or
 5 branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one acyl group; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one nitrogen containing moiety (e.g., nitro, nitrile, etc.); a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one amine subgroup; a linear or
 10 branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one ether subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one halogen subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one nitronium subgroup; wherein R5 is selected from the group
 15 consisting of: OH; NO₂; NR'; OR'; wherein R' is selected from the group consisting of: a linear or branched, saturated or unsaturated aliphatic chain having at least one carbon; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one hydroxyl subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one thiol
 20 subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, wherein said aliphatic chain terminates with an aldehyde subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one ketone subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons; wherein said aliphatic chain terminates with
 25 a carboxylic acid subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one amide subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one acyl group; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one nitrogen containing moiety
 30 (e.g., nitro, nitrile, etc.); a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one amine subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and

having at least one halogen subgroup; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons, and having at least one nitronium subgroup; wherein R6 is selected from the group consisting of: Hydrogen; NO₂; Cl; F; Br; I; SR'; and NR'₂; wherein R' is defined as above in R5; wherein R7 is selected from
5 the group consisting of: Hydrogen; a linear or branched, saturated or unsaturated aliphatic chain having at least 2 carbons; and wherein R8 is an aliphatic cyclic group larger than benzene; wherein said larger than benzene comprises any chemical group containing 7 or more non-hydrogen atoms, and is an aryl or aliphatic cyclic group. In some embodiments, R' is any functional group that protects the oxygen of R5 from
10 metabolism in vivo, until the compound reaches its biological target (e.g., mitochondria). In some embodiments, R' protecting group(s) is metabolized at the target site, converting R5 to a hydroxyl group.

EXAMPLES

15 The following examples are provided to demonstrate and further illustrate certain preferred embodiments of the present invention and are not to be construed as limiting the scope thereof.

Example 1

20 Preparation of Compounds

The benzodiazepine compounds are prepared using either solid-phase or soluble-phase combinatorial synthetic methods as well as on an individual basis from well-established techniques. *See, for example, Boojamra, C.G. et al. (1996); Bunin, B.A., et al. (1994); Stevens, S.Y. et al., (1996); Gordon, E.M., et al., (1994); and U.S.*
25 *Patent Nos. 4,110,337 and 4,076,823, which are all incorporated by reference herein. For illustration, the following general methodologies are provided.*

Preparation of 1,4-benzodiazepine-2-one compounds

Improved solid-phase synthetic methods for the preparation of a variety of 1,4-
30 benzodiazepine-2-one derivatives with very high overall yields have been reported in the literature. (*See e.g., Bunin and Ellman, J. Am. Chem. Soc., 114:10997-10998 [1992]*). Using these improved methods, the 1,4-benzodiazepine-2-ones is

constructed on a solid support from three separate components: 2-aminobenzophenones, α -amino acids, and (optionally) alkylating agents.

Preferred 2-aminobenzophenones include the substituted 2-aminobenzophenones, for example, the halo-, hydroxy-, and halo-hydroxy-substituted 2-aminobenzophenones, such as 4-halo-4'-hydroxy-2-aminobenzophenones. A preferred substituted 2-aminobenzophenone is 4-chloro-4'-hydroxy-2-aminobenzophenone. Preferred α -amino acids include the 20 common naturally occurring α -amino acids as well as α -amino acid mimicking structures, such as homophenylalanine, homotyrosine, and thyroxine.

Alkylating agents include both activated and inactivated electrophiles, of which a wide variety are well known in the art. Preferred alkylating agents include the activated electrophiles p-bromobenzyl bromide and t-butyl-bromoacetate.

In the first step of such a synthesis, the 2-aminobenzophenone derivative is attached to a solid support, such as a polystyrene solid support, through either a hydroxy or carboxylic acid functional group using well known methods and employing an acid-cleavable linker, such as the commercially available [4-(hydroxymethyl)phenoxy]acetic acid, to yield the supported 2-aminobenzophenone. (See e.g., Sheppard and Williams, Intl. J. Peptide Protein Res., 20:451-454 [1982]). The 2-amino group of the aminobenzophenone is preferably protected prior to reaction with the linking reagent, for example, by reaction with Fmoc-Cl (9-fluorenylmethyl chloroformate) to yield the protected amino group 2'-NHFMOC.

In the second step, the protected 2-amino group is deprotected (for example, the -NHFMOC group may be deprotected by treatment with piperidine in dimethylformamide (DMF)), and the unprotected 2-aminobenzophenone is then coupled via an amide linkage to an α -amino acid (the amino group of which has itself been protected, for example, as an -NHFMOC group) to yield the intermediate. Standard activation methods used for general solid-phase peptide synthesis are used (such as the use of carbodiimides and hydroxybenzotriazole or pentafluorophenyl active esters) to facilitate coupling. However, a preferred activation method employs treatment of the 2-aminobenzophenone with a methylene chloride solution of the α -N-Fmoc-amino acid fluoride in the presence of the acid scavenger 4-methyl-2,6-di-tert-butylpyridine yields complete coupling via an amide linkage. This preferred

coupling method has been found to be effective even for unreactive aminobenzophenone derivatives, yielding essentially complete coupling for derivatives possessing both 4-chloro and 3-carboxy deactivating substituents.

5 In the third step, the protected amino group (which originated with the amino acid) is first deprotected (*e.g.*, -NHFMOC may be converted to -NH₂ with piperidine in DMF), and the deprotected Bz-423s reacted with acid, for example, 5% acetic acid in DMF at 60°C, to yield the supported 1,4-benzodiazepine derivative. Complete cyclization has been reported using this method for a variety of 2-aminobenzophenone derivatives with widely differing steric and electronic properties.

10 In an optional fourth step, the 1,4-benzodiazepine derivative is alkylated, by reaction with a suitable alkylating agent and a base, to yield the supported fully derivatized 1,4-benzodiazepine. Standard alkylation methods, for example, an excess of a strong base such as LDA (lithium diisopropylamide) or NaH, is used; however, such methods may result in undesired deprotonation of other acidic functionalities and
15 over-alkylation. Preferred bases, which may prevent over-alkylation of the benzodiazepine derivatives (for example, those with ester and carbamate functionalities), are those which are basic enough to completely deprotonate the anilide functional group, but not basic enough to deprotonate amide, carbamate or ester functional groups. An example of such a base is lithiated 5-(phenylmethyl)-2-oxaxolidinone, which is reacted with the 1,4-benzodiazepine in tetrahydrofuran (THF)
20 at -78°C. Following deprotonation, a suitable alkylating agent, as described above, is added.

In the final step, the fully derivatized 1,4-benzodiazepine is cleaved from the solid support. This is achieved (along with concomitant removal of acid-labile
25 protecting groups), for example, by exposure to a suitable acid, such as a mixture of trifluoroacetic acid, water, and dimethylsulfide (85:5:10, by volume). Alternatively, the above benzodiazepines is prepared in soluble phase. The synthetic methodology was outlined by Gordon *et al.*, J. Med. Chem., 37:1386-1401 [1994]] which is hereby incorporated by reference. Briefly, the methodology comprises trans-imidating an
30 amino acid resin with appropriately substituted 2-aminobenzophenone imines to form resin-bound imines. These imines are cyclized and tethered by procedures similar to

those in solid-phase synthesis described above. The general purity of benzodiazepines prepared using the above methodology is about 90% or higher.

Preparation of 1,4-benzodiazepine-2,5-diones

5 A general method for the solid-phase synthesis of 1,4-benzodiazepine-2,5-diones has been reported in detail by C.J. Boojamra *et al.*, J. Org. Chem., 62:1240-1256 [1996]). This method is used to prepare the compounds of the present invention.

10 A Merrifield resin, for example, a (chloromethyl)polystyrene is derivatized by alkylation with 4-hydroxy-2,6-dimethoxybenzaldehyde sodium to provide resin-bound aldehyde. An α -amino ester is then attached to the derivatized support by reductive amination using NaBH(OAc)₃ in 1% acetic acid in DMF. This reductive amination results in the formation of a resin-bound secondary amine.

15 The secondary amine is acylated with a wide variety of unprotected anthranilic acids result in support-bound tertiary amides. Acylation is best achieved by performing the coupling reaction in the presence of a carbodiimide and the hydrochloride salt of a tertiary amine. One good coupling agent is 1-ethyl-8-[8-(dimethylamino)propyl] carbodiimide hydrochloride. The reaction is typically performed in the presence of anhydrous 1-methyl-2-pyrrolidinone. The coupling
20 procedure is typically repeated once more to ensure complete acylation.

 Cyclization of the acyl derivative is accomplished through base-catalyzed lactamation through the formation of an anilide anion which would react with an alkylhalide for simultaneous introduction of the substituent at the 1-position on the nitrogen of the heterocyclic ring of the benzodiazepine. The lithium salt of
25 acetanilide is a good base to catalyze the reaction. Thus, the Bz-423s reacted with lithium acetanilide in DMF/THF (1:1) for 30 hours followed by reaction with appropriate alkylating agent provides the fully derivatized support-bound benzodiazepine. The compounds are cleaved from the support in good yield and high purity by using TFA/DMS/H₂O (90:5:5).

30 Some examples of the α -amino ester starting materials, alkylating agents, and anthranilic acid derivatives that are used in the present invention are listed by Boojamra (1996), *supra* at 1246. Additional reagents are readily determined and

either are commercially obtained or readily prepared by one of ordinary skill in the art to arrive at the novel substituents disclosed in the present invention.

For example, from Boojamra, *supra*, one realizes that: alkylating agents provide the R₁ substituents; α -amino ester starting materials provide the R₂ substituents, and anthranilic acids provide the R₄ substituents. By employing these starting materials that are appropriately substituted, one arrives at the desired 1,4-benzodiazepine-2,5-dione. The R₃ substituent is obtained by appropriately substituting the amine of the α -aminoester starting material. If steric crowding becomes a problem, the R₃ substituent is attached through conventional methods after the 1,4-benzodiazepine-2,5-dione is isolated.

Example 2

Chirality

It should be recognized that many of the benzodiazepines of the present invention exist as optical isomers due to chirality wherein the stereocenter is introduced by the α -amino acid and its ester starting materials. The above-described general procedure preserves the chirality of the α -amino acid or ester starting materials. In many cases, such preservation of chirality is desirable. However, when the desired optical isomer of the α -amino acid or ester starting material is unavailable or expensive, a racemic mixture is produced which is separated into the corresponding optical isomers and the desired benzodiazepine enantiomer is isolated.

For example, in the case of the 2,5-dione compounds, Boojamra, *supra*, discloses that complete racemization is accomplished by preequilibrating the hydrochloride salt of the enantiomerically pure α -amino ester starting material with 0.3 equivalents of *i*-Pr₂EtN and the resin-bound aldehyde for 6 hours before the addition of NaBH(OAc)₃. The rest of the above-described synthetic procedure remains the same. Similar steps are employed, if needed, in the case of the 1,4-benzodiazepine-2-dione compounds as well.

Methods to prepare individual benzodiazepines are well-known in the art. (See e.g., U.S. 3,415,814; 3,384,635; and 3,261,828, which are hereby incorporated by reference). By selecting the appropriately substituted starting materials in any of

the above-described methods, the benzodiazepines of this invention are prepared with relative ease.

Example 3

Reagents

5 Bz-423 is synthesized as described above. FK506 is obtained from Fujisawa (Osaka, Japan). *N*-benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD) is obtained from Enzyme Systems (Livermore, CA). Dihydroethidium (DHE) and 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3)) are obtained from Molecular Probes
10 (Eugene, OR). FAM-VAD-fmk is obtained from Intergen (Purchase, NJ). Manganese(III)*meso*-tetrakis(4-benzoic acid)porphyrin (MnTBAP) is purchased from Alexis Biochemicals (San Diego, CA). Benzodiazepines is synthesized as described (See, B.A. Bunin *et al.*, Proc. Natl. Acad. Sci. U.S.A., 91:4708-4712 [1994]). Other reagents were obtained from Sigma (St. Louis, MO).

Example 4

Animals and drug delivery

Female NZB/W mice (Jackson Labs, Bar Harbor, ME) are randomly distributed into treatment and control groups. Control mice receive vehicle (50 µL
20 aqueous DMSO) and treatment mice receive Bz-423 dissolved in vehicle (60 mg/kg) through intraperitoneal injections. Peripheral blood is obtained from the tail veins for the preparation of serum. Samples of the spleen and kidney are preserved in either 10% buffered-formalin or by freezing in OCT. An additional section of spleen from each animal is reserved for the preparation of single cell suspensions.

Example 5

Primary splenocytes, cell lines, and culture conditions

Primary splenocytes are obtained from 6 month old mice by mechanical disruption of spleens with isotonic lysis of red blood cells. B cell-rich fractions are
30 prepared by negative selection using magnetic cell sorting with CD4, CD8a and CD11b coated microbeads (Miltenyi Biotec, Auburn, California). The Ramos line is purchased from the ATCC (Monassis, Georgia). Cells are maintained in RPMI

supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (290 µg/ml). Media for primary cells also contains 2-mercaptoethanol (50 µM). All *in vivo* studies are performed with 0.5% DMSO and 2% FBS. *In vitro* experiments are conducted in media containing
5 2% FBS. Organic compounds are dissolved in media containing 0.5% DMSO.

Example 6

Histology

Formalin-fixed kidney sections were stained with hematoxylin and eosin
10 (H&E) and glomerular immune-complex deposition is detected by direct immunofluorescence using frozen tissue stained with FITC-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL). Sections are analyzed in a blinded fashion for nephritis and IgG deposition using a 0-4+ scale. The degree of lymphoid hyperplasia is scored on a 0-4+ scale using spleen sections stained with
15 H&E. To identify B cells, sections are stained with biotinylated-anti-B220 (Pharmingen; 1 µg/mL) followed by streptavidin-Alexa 594 (Molecular Probes; 5 µg/mL). Frozen spleen sections are analyzed for TUNEL positive cells using an *In situ* Cell Death Detection kit (Roche) and are evaluated using a 0-4+ scale.

Example 7

TUNEL staining

Frozen spleen sections are analyzed using an *In situ* Cell Death Detection kit (Roche Molecular Biochemicals, Indianapolis, IN). Sections are blindly evaluated and assigned a score (0-4+) on the basis of the amount of TUNEL-positive staining.
25 B cells are identified by staining with biotinylated-anti-B220 (Pharmingen, San Diego, CA; 1 µg/mL, 1 h, 22 °C) followed by streptavidin-Alexa 594 (Molecular Probes, Eugene, Oregon; 5 µg/mL, 1 h, 22 °C).

Example 8

Flow cytometric analysis of spleen cells from treated animals

Surface markers are detected (15 m, 4 °C) with fluorescent-conjugated anti-Thy 1.2 (Pharmingen, 1 µg/mL) and/or anti-B220 (Pharmingen, 1 µg/mL). To detect

outer-membrane phosphatidyl serine, cells are incubated with FITC-conjugated Annexin V and propidium iodide (PI) according to manufacturer protocols (Roche Molecular Biochemicals). Detection of TUNEL-positive cells by flow cytometry uses the APO-BRDU kit (Pharmingen). Superoxide and MPT are assessed by incubation
5 of cells for 30 m at 27 degrees C with 10 μ M dihydroethidium and 2 μ M 3,3'-dihexyloxacarbocyanine iodide (DIOC₆(3)) (Molecular Probes). Propidium iodide is used to determine viability and DNA content. Samples are analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA).

10

Example 9

B cell stimulation

Ramos cells are activated with soluble goat Fab₂ anti-human IgM (Southern Biotechnology Associates, 1 μ g/ml) and/or purified anti-human CD40 (Pharmingen, clone 5C3, 2.5 μ g/ml). Mouse B cells are activated with affinity purified goat anti-
15 mouse IgM (ICN, Aurora, Ohio; 20 μ g/ml) immobilized in culture wells, and/or soluble purified anti-mouse CD40 (Pharmingen, clone HM40-3, 2.5 μ g/ml). LPS is used at 10 μ g/ml. Bz-423 is added to cultures immediately after stimuli are applied. Inhibitors are added 30 m prior to Bz-423.

20

Example 10

Statistical analysis

Statistical analysis is conducted using the SPSS software package. Statistical significance is assessed using the Mann-Whitney U test and correlation between variables is assessed by two-way ANOVA. All *p*-values reported are one-tailed and
25 data are presented as mean \pm SEM.

Example 11

Detection of cell death and hypodiploid DNA

Cell viability is assessed by staining with propidium iodide (PI, 1 μ g/mL). PI
30 fluorescence is measured using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA). Measurement of hypodiploid DNA is conducted after incubating cells in DNA-labeling solution (50 μ g/mL of PI in PBS containing 0.2% Triton and

10 $\mu\text{g/mL}$ RNase A) overnight at 4 degrees C. The data is analyzed using the CellQuest software excluding aggregates.

Example 12

5 **Detection of O_2^- , ψ_m , and caspase activation**

To detect O_2^- , cells are incubated with DHE (10 μM) for 30 min at 37 °C and are analyzed by flow cytometry to measure ethidium fluorescence. Flow analysis of mitochondrial transmembrane potential (ψ_m) is conducted by labeling cells with DiOC₆(3) (20 nM) for 15 min at 37 degrees C. A positive control for disruption of ψ_m is established using carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 50 μM). Caspase activation assays are performed with FAM-VAD-fluoromethylketone. Processing of the substrate is evaluated by flow cytometry.

Example 13

15 **Subcellular fractionation and cytochrome *c* detection**

Ramos cells (250×10^6 cells/sample) are treated with Bz-423 (10 μM) or vehicle for 1 to 5 h. Cells are pelleted, re-suspended in buffer (68 mM sucrose, 220 mM mannitol, 10 mM HEPES-NaOH, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 $\mu\text{g/mL}$ leupeptin, 10 $\mu\text{g/mL}$ aprotinin, 1 mM PMSF), incubated on ice for 20 10 min, and homogenized. The homogenate is centrifuged twice for 5 min at 4 °C (800g) to pellet nuclei and debris and for 15 min at 4 °C (16,000g) to pellet mitochondria. The supernatant is concentrated, electrophoresed on 12% SDS-PAGE gels, and transferred to Hybond ECL membranes (Amersham, Piscataway, NJ). After blocking (PBS containing 5% dried milk and 0.1% Tween), the membranes are 25 probed with an anti-cytochrome *c* monoclonal antibody (Pharmingen, San Diego, CA; 2 $\mu\text{g/mL}$) followed by an anti-mouse horseradish peroxidase-conjugated secondary with detection by chemiluminescence (Amersham).

Example 14

30 **ROS production in isolated mitochondria**

Male Long Evans rats are starved overnight and sacrificed by decapitation. Liver samples are homogenized in ice cold buffer A (250 mM sucrose, 10 mM Tris,

0.1 mM EGTA, pH 7.4), and nuclei and cellular debris are pelleted (10 min, 830g, 4 °C). Mitochondria are collected by centrifugation (10 min, 15,000g, 4 °C), and the supernatant is collected as the S15 fraction. The mitochondrial pellet is washed three times with buffer B (250 mM sucrose, 10 mM Tris, pH 7.4), and re-suspended in
5 buffer B at 20-30 mg/mL. Mitochondria are diluted (0.5 mg/mL) in buffer C (200 mM sucrose, 10 mM Tris, pH 7.4, 1 mM KH₂PO₄, 10 μM EGTA, 2.5 μM rotenone, 5 mM succinate) containing 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 1 μM). For state 3 measurements, ADP (2 mM) is included in the buffer, and prior to the addition of Bz-423, mitochondria are allowed to charge for 2 min. To induce state
10 4, oligomycin (10 μM) is added to buffer C. The oxidation of DCFH to 2',7'-dichlorofluorescein (DCF) is monitored at 37 °C with a spectrofluorimeter (λ_{ex} : 503 nm; λ_{em} : 522 nm). To detect effects on O₂⁻ and delta ψ_m , mitochondria are incubated for 15 min at 37 °C in buffer C with vehicle, Bz-423, or CCCP containing DHE (5 μM) or DIOC₆(3) (20 nM), and aliquots are removed for analysis by fluorescence
15 microscopy.

Example 15

Flow cytometric analysis of splenocytes

Splenocytes are prepared by mechanical disruption and red blood cells
20 removed by isotonic lysis. Cells are stained at 4 °C with fluorescent-conjugated anti-Thy 1.2 (Pharmingen; 1 μg/mL) and/or anti-B220 (Pharmingen; 1 μg/mL) for 15 min. To detect outer-membrane phosphatidyl serine, cells are incubated with FITC-conjugated Annexin V and PI (Roche Molecular Biochemicals, Indianapolis, IN; 1 μg/mL).

25

Example 16

In vivo determination of ROS

Spleens are removed from 4-mo old NZB/W mice treated with Bz-423 or vehicle and frozen in OCT. ROS production is measured using manganese(II)3,3,9-diaminobenzidine as described in E.D. Kerver *et al.* (See, E.D. Kerver *et al.*,
30 Histochem. J., 29:229-237 [1997]).

Example 17

IgG titers, BUN, and proteinuria

Anti-DNA and IgG titers are determined by ELISA as described in P.C.

- 5 Swanson *et al.* (See, P.C. Swanson *et al.*, Biochemistry, 35:1624-1633 [1996]).
Serum BUN is measured by the University of Michigan Hospital's clinical laboratory.
Proteinuria is monitored using ChemStrip 6 (Boehringer Mannheim).

Example 18

10 **Benzodiazepine studies**

Benzodiazepine studies on animals are described in U.S. Patent No.:
20010016583, published August 23, 2001, herein incorporated by reference in its
entirety.

15 **Example 19**

Mediators of Bz-423 induced apoptosis.

- To characterize the death mechanism engaged by Bz-423, intracellular ROS,
 $\Delta\Psi_m$, cytochrome *c* release, caspase activation, and DNA fragmentation were
measured over time (the results presented are for B cells but do characterize the
20 response in many different cell types). The first event detected after exposure to Bz-
423 is an increase in the fraction of cells that stain with dihydroethidium (DHE), a
redox-sensitive agent that reacts specifically with O_2^- .

- Levels of O_2^- diminished after an early maximum at 1 hour and then increased
again after 4 hours of continued treatment. This bimodal pattern pointed to a cellular
25 mechanism limiting O_2^- and suggested that the "early" and "late" O_2^- maxima resulted
from different processes.

Collapse of $\Delta\Psi_m$ was detected using DiOC₆(3), a mitochondria-selective
potentiometric probe. The gradient change began after the early O_2^- response and was
observed in >90% of cells by 5 hours.

- 30 Cytochrome *c* release from mitochondria, a key step enabling caspase
activation, was studied by immunoblotting cytosolic fractions. Levels of cytosolic
cytochrome *c* above amounts in cells treated with vehicle were detected by 5 hours.
This release was coincident with the disruption of $\Delta\Psi_m$, and together, these results

were consistent with opening of the PT pore. Indeed, the late increase in O_2^- tracked with the $\Delta\Psi_m$ collapse and the release of cytochrome *c*, suggesting that the secondary rise in O_2^- resulted from these processes.

Caspase activation was measured by processing of the pan-caspase sensitive fluorescent substrate FAM-VAD-fmk. Caspase activation tracked with $\Delta\Psi_m$, whereas the appearance of hypodiploid DNA was slightly delayed with respect to caspase activation. Collectively, these results indicated that Bz-423 induces a mitochondrial-dependent apoptotic pathway.

10

Example 20

Bz-423 directly targets mitochondria.

Since the early O_2^- preceded other cellular events, it was possible that this ROS had a regulatory role. In non-phagocytic cells, redox enzymes, along with the MRC, are the primary sources of ROS. Inhibitors of these systems were assayed for an ability to regulate Bz-423-induced O_2^- in order to determine the basis for this response. Of these reagents, only NaN_3 , which acts primarily on cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain, MRC), and micromolar amounts of FK506, which block the formation of O_2^- by the ubiquinol-cytochrome *c* reductase component of MRC complex III, modulated Bz-423. These findings suggested that mitochondria are the source of Bz-423-induced O_2^- and that a component of the MRC is involved in the response. Although the inhibition by FK506 may result from binding to either calcineurin or FK506-binding proteins, natural products that bind tightly to these proteins (rapamycin and cyclosporin A, respectively) did not diminish the Bz-423 O_2^- response.

O_2^- production by Bz-423 may result from binding to a protein within mitochondria or a target in another compartment that signals mitochondria to generate ROS. To distinguish between these alternatives, isolated rat liver mitochondria were assayed for ROS production by monitoring the oxidation of 2',7'-dichlorodihydrofluorescein diacetate to of 2',7'-dichlorofluorescein in the presence and absence of Bz-423. In this assay, the rate of DCF production increased after a lag period during which endogenous reducing equivalents were consumed and the acetate moieties on the probe were hydrolyzed to yield 2',7'-dichlorodihydrofluorescein, the

30

redox-active species. Under aerobic conditions supporting state 3 respiration, both antimycin A, which generates O_2^- by inhibiting ubiquinol-cytochrome *c* reductase, and Bz-423 increased the rate of ROS production nearly two-fold after the induction phase, based on comparing the slopes of each curve to control. Swelling was not
5 observed, demonstrating that Bz-423 does not directly target the MPT pore. Neither Bz-423 nor antimycin A generated substantial ROS in the subcellular S15 fraction (cytosol and microsomes), and Bz-423 does not stimulate ROS if mitochondria are in state 4, even though antimycin A is active under these conditions. Together, these experiments demonstrate that mitochondria contain a molecular target for Bz-423, and
10 state 3 respiration is required for the O_2^- response.

Example 21

Bz-423-induced ROS comes from mitochondria

MRC complexes I and III are the primary sources of ROS within
15 mitochondria. Evidence presented above suggests that Bz-423-induced ROS comes from mitochondria. To test this hypothesis, MRC function was knocked out the resulting cells were examined for ROS in response to Bz-423. Complexes I-IV in the MRC are partially encoded by mitochondrial DNA (mtDNA). Culturing cells over extended periods of time in the presence of ethidium bromide removed mtDNA,
20 suggesting that mtDNA encoded proteins are not produced and electron transport along the MRC does not occur (cells devoid of mtDNA and associated proteins are often termed ρ^0 cells). Because ethidium bromide is toxic to Ramos cells, these experiments were conducted with Namalwa B cells, another mature B cell line. Treating Namalwa ρ^0 cells with Bz-423 did not result in an ROS response, as was
25 observed in both Ramos and Namalwa ρ^+ cells.

Since the early ROS is critical to Bz-423 induced apoptosis, results detected with the Namalwa ρ^0 cells would seemingly predict that these cells would be protected from the toxic effects of Bz-423. However, after 6 hours, the MPT was triggered and Namalwa ρ^0 cells underwent apoptosis in response to Bz-423. In ρ^+
30 cells, proton pumping by the MRC maintained the mitochondrial gradient $\Delta\Psi_m$. Since a functional MRC is not present in ρ^0 cells, $\Delta\Psi_m$ is supported by complex V (the F_1F_0 -ATPase) functioning as an ATPase (deletion of subunits 6 and b in ρ^0 cells abolishes

the synthase activity of this enzyme). In this case, inhibition of complex V ATPase would cause collapse of the gradient and subsequent cell death.

Example 22

5 **Bz-423 targets the mitochondrial F_1F_0 -ATPase**

Oligomycin, a macrolide natural product that binds to the mitochondrial F_1F_0 -ATPase, induces a state 3 to 4 transition and generates O_2^- like Bz-423. Based on these similarities, it is possible that the F_1F_0 -ATPase is also the molecular target for Bz-423. To test this hypothesis, the effect of Bz-423 on ATPase activity in sub-
10 mitochondrial particles (SMPs) was examined. Indeed, Bz-423 inhibited the mitochondrial ATPase activity of bovine SMPs with an ED 50 ca. 5 μ M.

>40 derivatives of Bz-423 were developed to determine the elements on this novel agent required for biological activity. Assessing these compounds in whole cell apoptosis assays revealed that a hydroxyl group at the C'4 position and an aromatic
15 ring roughly the size of the naphthyl moiety were useful. The potency of these analogues in cell based assays correlated with the ED₅₀ values in ATPase inhibition experiments using SMPs. These observations indicated that the mitochondrial ATPase is the molecular target of Bz-423. At concentrations where these derivatives are cytotoxic (80 μ M), other benzodiazepines and PBR ligands (e.g., PK11195 and 4-
20 chlorodiazepam) do not significantly inhibit mitochondrial ATPase activity, suggesting that the molecular target of Bz-423 is distinct from the molecular target(s) of these other compounds.

Example 23

25 **Bz-423 binds to the OSCP**

As part an early group of mechanistic studies of Bz-423, a biotinylated analogue was synthesized by replacing the N-methyl group with a hexylaminolinker to which biotin was covalently attached (this modification did not alter the activity of Bz-423). This molecule was used to probe a display library of human breast cancer
30 cDNAs (Invitrogen) that are expressed as fusion proteins on the tip of T7 phage. Following the screening methods described by Austin and co-workers using biotinylated version of KF506 to identify new FK506 binding proteins, the OSCP

component of the mitochondrial F_1F_0 -ATPase was identified as a binding protein for Bz-423 (Figure 1).

To determine if Bz-423 indeed binds to the OSCP and the affinity of the interaction, human OSCP was overexpressed in *E. coli*. Titrating a solution of Bz-423 into the OSCP resulted in quenching of the intrinsic protein fluorescence and afforded a K_d of 200 ± 40 nM (Figure 2). The binding of several Bz-423 analogues was also measured and it was found that their affinity for the OSCP paralleled their potency in both whole cell cytotoxicity assays as well as ATPase inhibition experiments using SMP. These data provided cogent evidence that Bz-423 binds to the OSCP on the mitochondrial ATPase. Bz-423 is the only known inhibitor of the ATPase that functions through binding to the OSCP. Since the OSCP does not contain the ATP binding site and it does not comprise the proton channel, it is possible that Bz-423 functions by altering the molecular motions of the ATPase motor.

Example 24

RNAi knockouts of the OSCP protect against Bz-423 induced cell death

To complement the chemical and biochemical target identification and validation studies described above, experiments were conducted to knockout the OSCP in whole cells. In vitro, removing the OSCP from the ATPase abolishes synthase function without altering the hydrolytic activity of the enzyme. In yeast, OSCP knockouts are not lethal; in these cells, hydrolysis of ATP provides the chemical potential to support $\Delta\Psi_m$ thereby maintaining mitochondrial integrity. Since yeast OSCP has limited sequence homology to the mammalian protein (~30%), these experiments were conducted in cell lines from human origin.

Since the OSCP is nuclear encoded, RNA interference (RNAi), a technique that can achieve post-transcriptional gene silencing, was employed to knockout this protein. For these experiments, HEK 293 cells were transfected with each of three chemically synthesized small interfering RNA molecules (siRNA) specific for the OSCP sequence using oligofectamine. These cells are transfected in a highly efficient (90%) manner by oligofectamine. OSCP expression was analyzed by immunoblot at 24h, 48h, 72h and 96h after transfection. The maximum silencing of OSCP expression (64%) occurred at 72h after transfection (Figure 3). OSCP siRNA transfected HEK

293 cells had a reduced Bz-ROS and apoptosis in response to Bz-423 relative to cells transfected with a scrambled sequence control siRNA. These results indicated that siRNA is effective at reducing OSCP and suggested that Bz-423 mediated cell death signaling involves the OSCP.

5

Example 25

Effect of Bz-423 on cellular proliferation

Like most 1,4-benzodiazepines, Bz-423 binds strongly to bovine serum albumin (BSA), which reduces the effective concentration of drug free in solution.

10 For example, in tissue culture media containing 10% (v/v) fetal bovine serum (FBS), ca. 99% of the drug is bound to BSA. Therefore, cell culture cytotoxicity assays are conducted in media with 2% FBS to reduce binding to BSA and increase the free [Bz-423]. Under these conditions, the dose response-curve is quite sharp such that there is a limited concentration range at which Bz-423 is only partly effective. Since some

15 benzodiazepines are known to have anti-proliferative properties, the effect of Bz-423 at concentrations $< ED_{50}$ were carefully analyzed and observed that in addition to inducing apoptosis, Bz-423 prevented cell growth after 3 d in culture. In these low serum conditions, the cytotoxic and anti-proliferative effects overlapped making it difficult to study each effect independently. However, by increasing the [BSA] or

20 increasing FBS to 10%, the dose-response curve flattened (and the cytotoxicity ED_{50} increased) and Bz-423 induced cytotoxicity could be clearly distinguished from effects on proliferation. At lower amounts of drug (*e.g.*, 10-15 μM), Bz-423 had minimal cytotoxicity whereas at concentrations $> 20 \mu M$ only apoptosis was observed (the death pathway described above including a bimodal ROS response, and was also

25 observed in media containing 10% FBS). While higher amounts of drug may also block proliferation, it caused apoptosis well before the effects on proliferation could be observed. Dose response curves were similar in experiments where BSA was added to media containing 2% FBS to simulate media containing 10% FBS, which demonstrated that antiproliferation and cytotoxicity were not affected by other

30 constituents of serum.

To confirm the decrease in cell number relative to control cells after 3 d of treatment is due to decreased proliferation and not cell death balanced by

proliferation, in addition to cell counting, cell divisions were studied. PKH-67 is a fluorescent probe that binds irreversibly to cell membranes and upon cell division is partitioned equally between the daughter cells, making it possible to quantify cell division by flow cytometry. Ramos cells stained with PKH67 and treated with Bz-423 had fewer cell divisions at sub-cytotoxic concentrations which confirmed that the decrease in cell number was due to anti-proliferative effects and not cell death. To determine if Bz-423 induced anti-proliferation was specific to Ramos cells, cell counting and cell cycle experiments were done in other B cell lines and cell lines derived from solid tumors. As seen in Table 3, the effects on blocking proliferation were not unique to lymphoid cells which suggested a target, common to multiple tissue types, mediated the block in proliferation.

Table 3. ED₅₀ (μM) for antiproliferation of cells treated for 72 h in media with 10% FBS. Cells for study included Ramos cells and clones transfected to overexpress Bcl-2 and Bcl-x_L, ovarian cells with null p53 (SKOV3); neuroblastoma cell lines (IMR-32, Lan-1, SHEP-1); and malignant B cell lines.

Ramos	Bcl-2	Bcl-X _L	SKOV3	IMR-32	Lan-1	SHEP-1	CA46	Raji
10.7	11.9	13.7	18.2	18.0	13.7	15.9	13.4	12.9

Example 26

Gene profiling cells treated with Bz-423.

Gene profiling experiments were conducted to probe the mechanism by which Bz-423 blocks cellular proliferation. In studies using cyclohexamide as an inhibitor of protein synthesis, it was found that Bz-423-induced cell death did not depend on new protein synthesis. Therefore, changes in gene expression were more likely relevant only to the mechanism of anti-proliferation. To increase the likelihood of detecting changes involved in signal-response coupling rather than down-stream effects, cells were profiled that were treated with Bz-423 for 3 h. This is the point just after the ROS early maximum, but before other cellular changes occur, including opening of the mitochondria permeability pore.

The discovery of the pro-apoptotic, cytotoxic and growth inhibitory properties of Bz-423 against pathogenic cell types identified the potential for this class of agents to be therapeutic against autoimmune diseases, cancers and other neoplastic diseases. Further experimental evidence from an analysis of the changes in gene expression

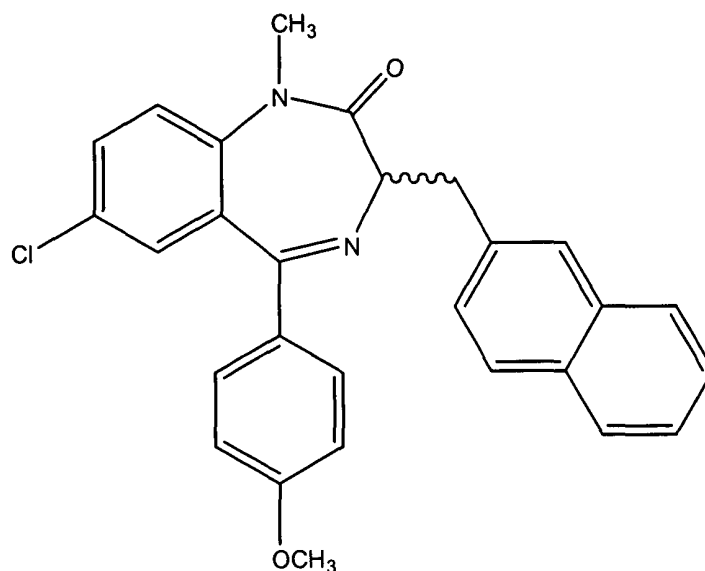
induced by this agent expanded the mechanistic understanding of this compound's action and added to the collection of therapeutic effects it modulates.

In vitro testing with Ramos cells to determine the changes in gene expression (at the level of mRNA) induced by Bz-423 was performed by culturing cells at a density of 500,000 cells per ml. Solvent control (DMSO, final concentration 0.1% V/V), Bz-423, or Bz-OMe (10 μ M) was added to cells. After 4 h, cells were harvested and RNA prepared using Trizol Reagent (#15596-018, Life Technologies, Rockville, MD) and the RNeasy Maxi Kit (# 75162, Qiagen, Valencia, CA) according to manufacturers protocols. Single stranded cDNA was synthesized by reverse transcription using poly (A) RNA present in the starting total RNA sample. Single stranded cDNA was converted into double stranded cDNA and then in vitro transcription carried out in the presence of biotinylated UTP and CTP to produce biotin-labeled cRNA. cRNA was fragmented in the presence of Mg²⁺, and hybridized to the human genome U133A Genechip array (Affymetrix). Hybridization results were quantified using a GeneArray scanner and analysis carried out according to the instructions provided by Affymetrix.

Expression profiling using RNA isolated from cells treated with Bz-423, Bz-OMe, or vehicle control was done with the HGU133A Affymetrix gene chip, which represents about 22,000 human genes. Using criteria that include $p < 0.01$, 16 genes are expressed 8-fold or more over control cells. As expected based on the molecular target of Bz-423, many of these genes were involved in glycolysis.

The data were analyzed to detect genes changes Bz treatment according to the criteria that the log-transformed mean signal changed at least four-fold in treated compared to vehicle control samples and that the coefficient of variance for control values ($n=4$) was less than 10%. These genes represent targets that may mediate therapeutic responses.

The gene expression results for Bz-423 and Bz-OMe each provide a unique fingerprint of information. The structure of Bz-OMe is as follows:



Expression of some genes change similarly after exposure to both Bz-423 and Bz-OMe. Thus, the genes that are commonly regulated between the two compounds are particularly relevant for understanding gene regulation through a more general class of compounds. Figure 4 presents data showing gene expression profiles of cells treated by Bz-423 and Bz-OMe.

Example 27

Effect of Bz-423 on ODC levels and activity

To determine whether ODC activity and polyamine metabolism is affected by Bz-423, as suggested by RNA profiling data, ODC activity in cells treated with Bz-423 was directly measured in comparison with a vehicle control. In these experiments, the conversion of ornithine to putrescine was quantified using ^3H -ornithine. For comparisons, control cells were treated with vehicle control or difluoromethyl ornithine (DFMO), a potent inhibitor of ornithine decarboxylase (like Bz-423, DFMO is a potent anti-proliferative agent). As seen in Figure 3, treating cells for 4 h with Bz-423 significantly reduced ODC activity in a dose-dependant fashion, which is consistent with among other things, an increase in antizyme 1, as suggested by RNA profiling. The reduction in ODC activity was paralleled by a decrease in ODC protein levels measured under the same conditions.

As described above, Bz-423 induced apoptosis was signaled by an ROS response that arose from MRC complex III as a result of the state 3 to 4 transition. It was next sought to determine if the ROS response, critical for apoptosis, also mediated these effects on ODC. If the ROS was required for the decrease in ODC activity, it would likewise be implicated as potentially part of the anti-proliferative response to Bz-423. To test this, Ramos cells were treated with Bz-423, DFMO, or vehicle control for 4 h. In parallel, a second group of cells was pre-incubated with MnTBAP to limit the ROS and then cultured with Bz-423, DFMO, and vehicle control. MnTBAP significantly reversed inhibition of ODC by Bz-423.

Collectively these data suggested the possible interpretation that high [Bz-423] (e.g. >10 μ M) generate sufficient amounts of ROS that could not be detoxified by cellular anti-oxidants, and resulted in apoptosis within 18 h. Lower [Bz-423] induced a proportionally smaller ROS response that was insufficient to trigger apoptosis. In this case, however, the ROS may be capable of inhibiting ODC or otherwise blocking cellular proliferation.

Consistent with this hypothesis, a compound in which the phenolic hydroxyl is replaced by Cl (designated Bz-Cl) was minimally cytotoxic (activity decreased by ca 80% compared to Bz-423) and generated a small ROS response in cells, while also binding less tightly to the OSCP ($K_d \approx 5 \mu$ M). This compound also inhibited ODC activity (Figure 3), as predicted by the above hypothesis. Given the proposed role and nature of Bz-423 induced ROS in mediating growth arrest, Bz-Cl was tested against the panel of cells in Table 2 and found that after 3 d it reduced proliferation to a similar extent as Bz-423, with comparable ED_{50} values. These results demonstrated that the antiproliferative effects of these compounds could be obtained using chemical analogues of Bz-423 that block proliferation without inducing apoptosis.

Example 28

Structure Activity Studies of Novel Cytotoxic Benzodiazepines

Based on these properties of Bz-423, a range of Bz-423 derivatives were synthesized to probe structural elements of this novel compound important for binding and activity. Replacing the N-methyl group or chlorine with a hydrogen had little effect on lymphotoxic activity against immortalized Ramos B cells or Jurkat T cells in

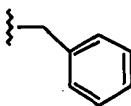
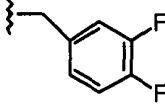
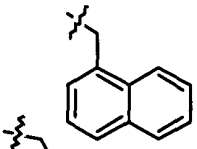
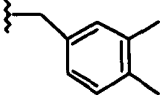
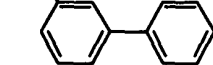
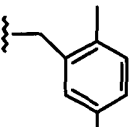
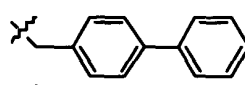
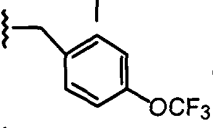
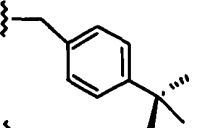
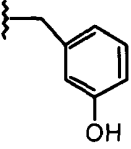
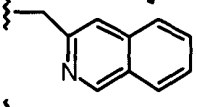
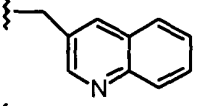
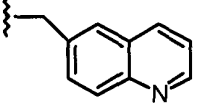
culture. Similarly, both enantiomers of Bz-423 were equipotent, which indicates that the interaction between Bz-423 and its molecular target involves two-point binding. In contrast to these data, removing a naphthalalanine (see Table 1). The present invention is not limited to a particular mechanism, and an understanding of a

5 mechanism is not necessary to practice the present invention, nonetheless, it is contemplated that moiety or replacing the phenolic hydroxyl group with hydrogen abolished all cytotoxic activity (Table 1). Based on these observations changes to the C'3 and C'4 positions were investigated. Replacing 1-naphthol with 2-naphtho has little effect on cell killing. Similarly, replacing the naphthylalanine with other

10 hydrophobic groups of comparable size had little effect on cytotoxic properties of Bz-423. By contrast, quinolines 7-9 were each less potent than Bz-423. The present invention is not limited to a particular mechanism, and an understanding of a mechanism is not necessary to practice the present invention, nonetheless, it is contemplated that theses data suggest a preference for a hydrophobic substituent

15 within the binding site for Bz-423. Smaller C3 substituents were only somewhat less potent than Bz-423 whereas compounds with aromatic groups containing oxygen were significantly less cytotoxic. These data clearly indicate that a bulky hydrophobic aromatic substituent is useful for optimal activity.

Table 1. Potency of Bz-423 derivatives. Cell death was assessed by culturing Ramos B cells in the presence of each compound in a dose-response fashion. Cell viability was measured after 24 h propidium iodide exclusion using flow cytometry. In this assay, the EC₅₀ for PK11195, diazepam, and 4-Cl-diazepam is > 80 μM.

	Compound	EC ₅₀ (μM) ^a		Compound	EC ₅₀ (μM)
– naphthalAla	1	>80		10	12
– phenol	2	>80		11	10
	3	5		12	6
	4	4		13	7
	5	7		14	35
	6	4		15	25
	7	11			
	8	12			
	9	15			

^aEach EC₅₀ value was determined twice in triplicate and has an error of ±5%.

Placing a methyl group ortho to the hydroxyl (16) does not alter the activity of Bz-423 whereas moving the hydroxyl to the C'4 (17) position decreased potency 2-fold (Table 2). By contrast, replacing the hydroxyl with chlorine or azide, or

5 methylating the phenol effectively abolishes the cytotoxic activity of Bz-423. The present invention is not limited to a particular mechanism, and an understanding of

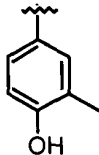
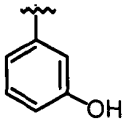
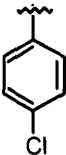
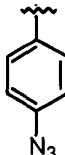
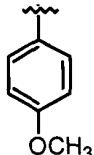
the mechanism is not necessary to practice the present invention, nonetheless, it is contemplated that these data indicate that a hydroxyl group positioned at the C'4 carbon is required for optimal activity, possibly by making a critical contact upon target binding. However, molecules possessing a phenolic substructure can also act as

5 alternate electron carriers within the MRC. Such agents accept an electron from MRC enzymes and transfer it back to the chain at point of higher reducing potential. This type of 'redox cycling' consumes endogenous reducing equivalents (e.g., glutathione) along with pyrimidine nucleotides and results in cell death. To distinguish between these alternatives, it was determined whether Bz-423 redox cycles in the presence of

10 sub-mitochondrial particles using standard NADH and NAD(P)H oxidation assays. Unlike the positive controls (doxorubicin and menadione), Bz-423 does not lead to substrate oxidation which strongly suggests that it does not redox cycle. The present invention is not limited to a particular mechanism, and an understanding of the mechanism is not necessary to practice the present invention, nonetheless, it is

15 contemplated that collectively, the data indicate that the decreased activity of compounds 18-20 results from removing an interaction that mediates binding of Bz-423 to its target protein.

Table 2. Potency of Bz-423 derivatives. Cell death was assessed as described in Table 1

					
Compound	16	17	18	19	20
EC₅₀	3	6	>80	>80	>80

20

Cells rapidly produce O_2^- in response to Bz-423 and blocking this signal (e.g., by inhibiting ubiquinol cytochrome *c* reductase, which is the enzyme that produces O_2^- in response to Bz-423) prevents apoptosis. To determine if the Bz-423 derivatives kill cells in manner analogous to Bz-423 (presumably as a result of binding to a

common molecular target), the ability of FK506 was examined, micromolar amounts of which effectively inhibit ubiquinol cytochrome *c* reductase, to protect against cell death. Inhibition by FK506 (\approx 60%) was only observed for 3-6, 12, 13, 16, and 17, which are the compounds with hydrophobic C3 side chains larger than benzene. Cell death induced by each of these compounds (including Bz-423) was also inhibited (to \approx 60%) by pre-treating cells with either 18, 19, or 20 (at $> 40 \mu\text{M}$). Compounds 18, 19, and 20 had no effect on blocking the cytotoxic activity (inhibition of \approx 20%) of the other benzodiazepines listed in Table 2. The present invention is not limited to a particular mechanism, and an understanding of the mechanism is not necessary to practice the present invention, nonetheless, it is contemplated that these data strongly suggest that Bz-423 along with 3-6, 12, 13, 16, and 17 bind the same site within the target protein and induce apoptosis through a common mechanism. The other compounds do not bind at this site and induce a death response through a different pathway.

15 All publications and patents mentioned in the above specification are herein incorporated by reference. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.